UNIVERSITÉ DU QUÉBEC

MÉMOIRE PRÉSENTÉ À

L'UNIVERSITÉ DU QUÉBEC À TROIS-RIVIÈRES

COMME EXIGENCE PARTIELLE

DE LA MAÎTRISE EN SCIENCES DE L'ENVIRONNEMENT

PAR

CAROLE CHARBONNEAU

EFFETS *IN VIVO* ET *IN VITRO* DE L'AZADIRACHTINE ET DE MOLÉCULES ÉPOXY-ALCOOL SIMPLES SUR COLÉOPTÈRES, DIPTÈRES ET LÉPIDOPTÈRES

JANVIER 2007

AVANT-PROPOS

Conformément aux articles 136 à 138 du règlement des études de cycles supérieurs de l'Université du Québec à Trois-Rivières, il est possible de présenter les résultats obtenus dans le cadre du programme de 2^{e} cycle en sciences de l'environnement sous forme d'articles scientifiques.

Le chapitre I, du présent document, expose un résumé substantiel incluant une introduction détaillée du sujet dans laquelle sont présentés la problématique ainsi que les objectifs et la méthodologie du travail de même que les résultats obtenus, les conclusions et une liste des références.

Le chapitre II présente le premier article scientifique traitant la partie *in vivo* du travail réalisé et a pour titre "Effects of azadirachtin and simpler epoxy-alcohols on survival and behaviour of *Galleria mellonella* (Lepidoptera) larvae". Les auteurs sont Carole Charbonneau, Roland Côté et Guy Charpentier. Le document a été soumis au *Journal of Applied Entomology* pour fin de publication. Les instructions relatives à la publication d'articles dans ce journal sont présentées en annexe.

Le chapitre III présente le deuxième article scientifique traitant cette fois la partie *in vitro* de l'étude réalisée et a pour titre "Compared effects of azadirachtin and simpler epoxy-alcohols on insect cell Coleoptera, Diptera and Lepidoptera". Les auteurs sont Carole Charbonneau, Roland Côté et Guy Charpentier. Le papier a été soumis au *Entomologia Experimentalis et Applicata* pour fin de publication. Les instructions à l'intention de l'auteur en vue de publier dans ce journal se retrouve en annexe.

REMERCIEMENTS

L'aboutissement d'un tel travail n'aurait pu avoir lieu sans la participation de certaines personnes et organismes que je tiens à souligner.

Je tiens à remercier dans un premier temps mon directeur de recherche, le docteur Guy Charpentier qui a su me guider dans la réalisation de mes travaux. Je le remercie également pour sa grande compréhension sans laquelle la poursuite de mes études n'aurait pu être possible. Un grand merci également à mon co-directeur le docteur Roland Côté pour ses judicieux conseils.

Des remerciements sont également adressés à Caroline Descôteaux pour la synthèse des produits, à Madame Hélène Colas-Charpentier pour son aide précieuse concernant la correction de l'anglais des deux articles sans oublier toute l'équipe du laboratoire dont Christine Gaudreau, Lise Meilleur et Mylène St-Onge.

Pour l'aide financière apportée, je tiens à remercier les Fonds Québécois de la recherche sur la Nature et les Technologies ainsi que la Fondation de l'Université du Québec à Trois-Rivières.

Finalement pour le soutien moral et les encouragements qu'ils m'ont manifestés tout au long de mes études universitaires, je remercie ma famille, mon conjoint et mes enfants.

TABLE DES MATIÈRES

LISTE DES TABLEAUX

LISTE DES FIGURES

LISTE DES SYMBOLES ET ABRÉVIATIONS

Aza	Azadirachtine, Azadirachtin
°C	Degré Celsius
CH_2Cl_2	Dichlorométhane
CL_{50}	Concentration létale 50 %
DDT	Dichloro diphényl trichloroéthane
DL_{50}	Dose létale 50 %
IGR	Insect growth regulator
g	Gramme, gram
h	Heure, hour
l	Litre
LD_{50}	Lethal dose 50%
Log	Logarithme
m	Milli 10^{-3}
MCPBA	Acide méta-chloroperbenzoïque
Min	Minute
ppm	Partie par million, part per million
RMN-^{13}C	Résonance magnétique nucléaire de carbone
RMN-^{1}H	Résonance magnétique nucléaire d'hydrogène
μ	Micro 10^{-6}
UV	Ultraviolet, UV light

CHAPITRE I

RÉSUMÉ

INTRODUCTION

Problématique

L'objectif général de la présente recherche cadre parfaitement avec la pensée écologique actuelle et la vague d'intérêt qu'on connaît aujourd'hui pour les biopesticides d'origine végétale. Cette nouvelle demande influence la recherche de telle sorte que cette dernière tend désormais vers un contrôle biologique des insectes nuisibles. Jusqu'à tout récemment, l'épandage de pesticides chimiques est apparu comme le moyen le plus efficace et le moins coûteux de contrôler les organismes nuisibles. Outre les bénéfices retirés (augmentation des rendements, protection des réserves alimentaires, lutte contre les vecteurs de maladies, lutte contre les parasites ou encore la protection de certaines espèces), ces modifications chimiques que l'espèce humaine impose aux écosystèmes naturels, ont des effets non seulement sur le milieu visé mais également sur les écosystèmes adjacents et par rétroaction, sur elle-même. Ces pesticides, du fait de leurs natures chimiques, sont polluants, toxiques et cancérogènes et nombre d'effets néfastes découlant de leur utilisation ont été répertoriés, à savoir la non spécificité des produits (Riba et Silvy, 1989), l'augmentation du phénomène de résistance chez les insectes (Bliefert et Perraud, 2001), la pollution des eaux (Painchaud, 1999), la consommation d'aliments contaminés (Doucet-Leduc, 1991) ou encore l'impact sur la santé (Tron *et al.*, 2001).

Sachant que quatre-vingts pour cent des êtres vivants occupant la planète sont des insectes (Bourassa, 2000) et que de ce nombre près de dix milles catégories d'insectes sont reconnues nuisibles pour l'homme (Ware, 2000), il est toujours d'actualité de mener une lutte efficace contre ces pestes (Vincent et Coderre, 1992). Toutefois, suite à l'interdiction de l'utilisation (Ministère de l'environnement, 2003) et de la chute de popularité des insecticides chimiques classiques, il est essentiel de proposer de nouvelles options respectueuses de l'environnement et efficaces pour la lutte contre les insectes nuisibles.

Parmi les interventions en faveur d'une lutte biologique, il y a notamment l'utilisation d'insecticides botaniques. De nombreuses plantes produisent naturellement diverses substances à effets toxiques antiappétants ou intervenant dans la régulation de la croissance des insectes (Riba et Silvy, 1989). Plus de deux milles plantes appartenant à plus de soixante familles ont été reconnues par Dev et Koul (1997) comme ayant un pouvoir insecticide.

Les premières générations d'insecticides végétaux (Figure1.1) sont essentiellement le résultat de l'utilisation de produits facilement disponibles. Parmi les composés d'origine végétale utilisés comme répulsifs ou produits toxiques; les alcaloïdes extraits du tabac agissent à la fois comme poison cardiaque et neurotrope, les roténoïdes extraient des racines de *Derris elliptica* agissent sur les mécanismes de la respiration cellulaire et les pyréthrines isolées des plantes appartenant à la famille des Astéracées, agissent en perturbant la conduction nerveuse ayant comme conséquence chez l'insecte une hyperactivité suivie de convulsions (effet "knock down").

Quoi que botaniques, ces insecticides n'en demeurent pas moins toxiques pour certaines espèces non ciblées. La nicotine par exemple, très toxique pour les mammifères avec une dose mortelle (DL_{50}) pour l'homme de l'ordre de 50-60 mg per os, a vu son emploi, comme produit phytosanitaire, considérablement limité. Aussi, il y a la roténone qui est inoffensive chez les animaux à sang chaud mais très nocive chez ceux à sang froid comme les batraciens, les poissons et les reptiles. L'instabilité moléculaire est aussi une contrainte qui a conduit à la synthèse de molécules plus stables comme la deltaméthrine et le fenvalérate deux dérivées des pyréthrines.

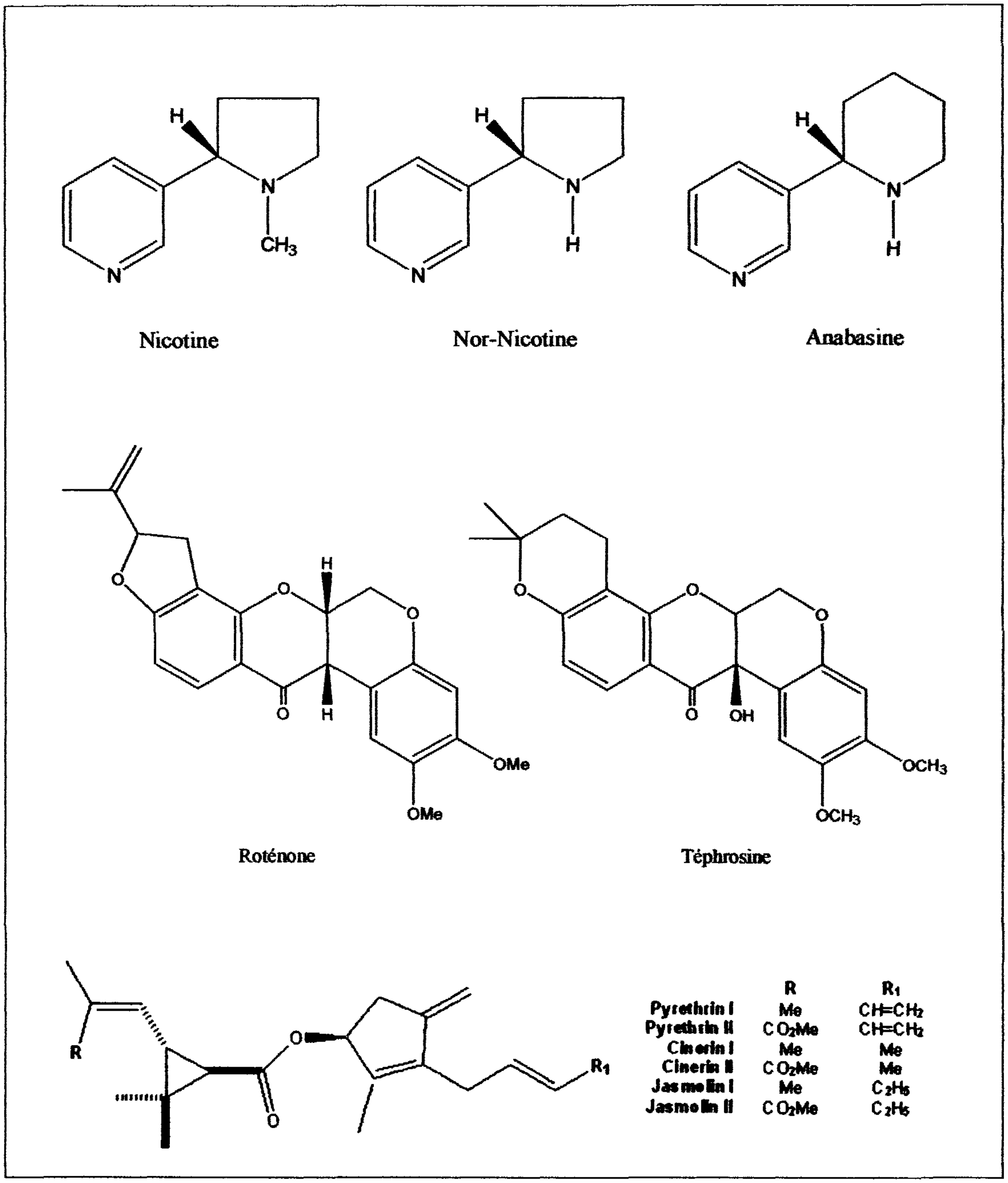
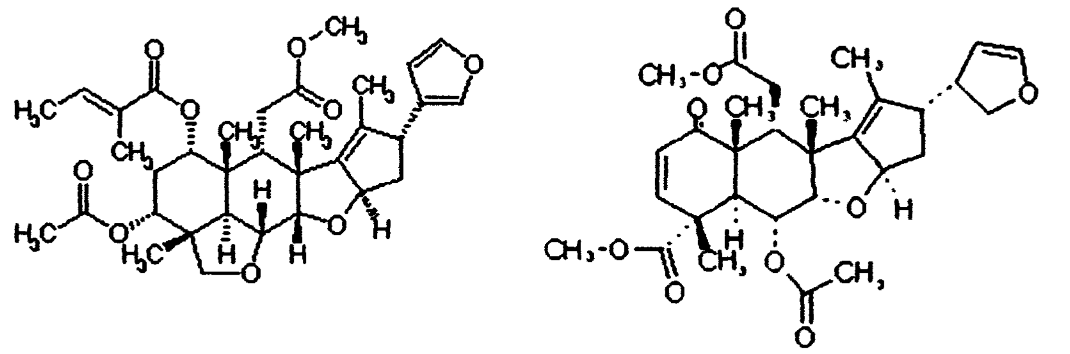


FIGURE 1.1 Insecticides biologiques d'origine végétale. Sont présentés, premièrement les alcaloïdes extraits du tabac, la nicotine, l'anabasine et la nor-nicotine; ensuite les roténoïdes, la roténone extraite des racines de *Derris elliptica* et la téphrosine extraite du *Tephrosia virginiana*. Finalement les pyréthrinoïdes où R et R_1 représentent les différents substituants de la molécule.

Aujourd'hui les pyréthrinoïdes de synthèse sont de loin les plus connus et les plus utilisés. Toutefois, le retour vers des molécules d'origine végétale a permis aux scientifiques de redécouvrir une plante remarquable qui a retenu l'attention et a fait l'objet d'innombrables recherches et publications; le margousier de la famille des *Meliacae* ou arbre de neem (*Azadirachta indica* A. Juss). Son utilisation en Inde, d'où il origine, fait partie des pratiques traditionnelles depuis plus de quatre milles ans (Larson, 1989). De nos jours, le neem se retrouve sur le marché mondial sous différentes formulations notamment Margosan-O®, le premier produit à base de neem à avoir été commercialisé (Schmutterer et Ascher, 1987). L'huile de neem compte dans sa composition un minimum de trente-cinq composés biologiquement actifs (Mulla et Su, 1999). Tous ces composés ne présentent d'ailleurs pas la même activité. Le neem doit son pouvoir insecticide principalement à des composés limonoïdes comme la salanine, la nimbine (Figure 1.2), et particulièrement l'azadirachtine (Figure 1.3). La salanine et la nimbine sont des antiappétants. Seule l'azadirachtine agit aussi comme inhibiteur de croissance (Isman, 1997).



a) b)

FIGURE 1.2 Structure des composés limonoïdes extraits de l'huile de neem. La molécule a correspond à la structure de la salanine tandis que le composé b représente la nimbine.

C'est Butterworth et Morgan, (1968,1971) qui ont été les premiers à isoler la substance active et c'est finalement en 1985 que la structure complète de l'azadirachtine (Figure 1.3) a été élucidée (Krauss *et al.*, 1985 ; Broughton *et al.*, 1986).

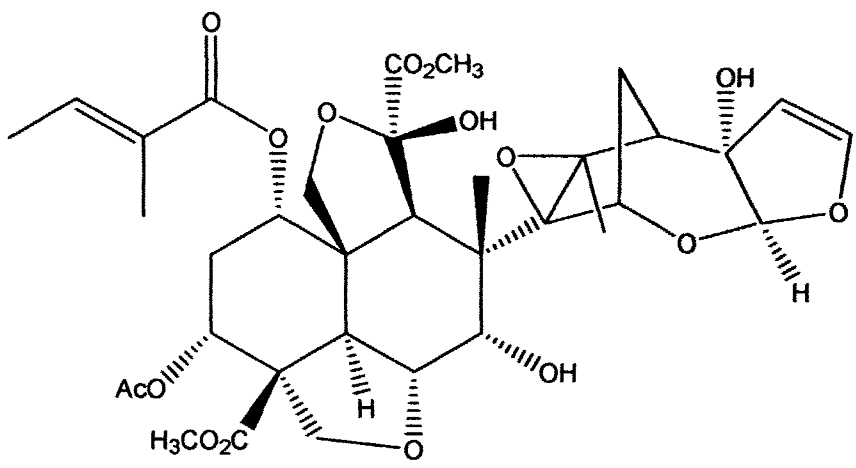


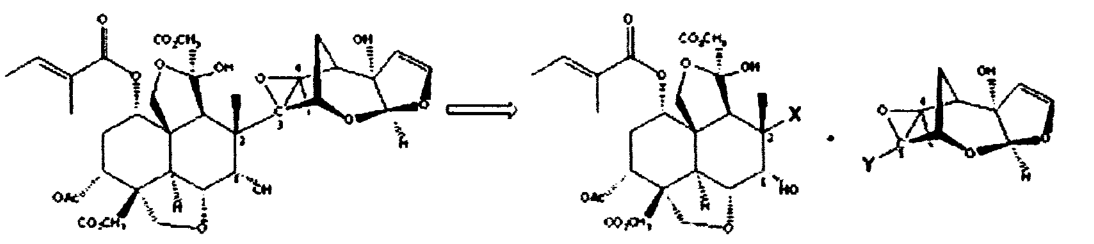
FIGURE 1.3 Composé majoritaire extrait de l'huile de neem: azadirachtine (Aza A). Parmi la douzaine d'analogues à l'azadirachtine, seulement deux composés, l'azadirachtine (Aza A) et le 3-tigloylazadirachtol (Aza B) présente une activité significative. Le composé Aza B se retrouve dans une proportion ne dépassant pas vingt-cinq pour cent par rapport au composé Aza A et tous les autres analogues ne totalisent pas plus de cinq pour cent. Dans le présent document l'appellation azadirachtine fera référence uniquement à l'Aza A ci-haut.

Outre son grand pouvoir insecticide, l'azadirachtine est très sélective envers certaines classes d'insectes. Ce bioinsecticide est également sans danger pour les mammifères et les oiseaux. Utilisée aux doses efficaces, elle ne présente aucun risque important pour la plupart des organismes non ciblés comme les abeilles, les poissons et les insectes aquatiques (Scott et Kaushik, 1998). Testée sur plus de quatre cent espèces d'insectes parmi les Noctuidae, les

Cicadidae, les Chrysomelidae, les Tortricidae et les Curculionidae, l'azadirachtine a manifesté une activité, qui s'accompagne d'une grande variabilité des doses létales cinquante pourcent (DL_{50}), sur plus de quatre-vingt-dix pour cent d'entre elles (Koul, 1999). L'azadirachtine agit comme antiappétant (Darazy-Choubaya, 2002) à des concentrations variant de dix à cent partie par million (ppm) selon l'espèce et principalement comme inhibiteur de croissance ou *Insect Growth Regulator* (IGR) à des concentrations variant de un à dix ppm (Govindachari *et al.,* 1996). D'autres effets provoqués par l'azadirachtine ont été rapportés, notamment une diminution de la fécondité. Selon Moreira (1994), un traitement à l'azadirachtine limite la capacité des femelles à produire des œufs. Une diminution de l'oviposition est également observée. Selon Su et Mulla (1999) un traitement à l'azadirachtine, à une concentration de cinq à dix ppm, sur des populations de *Culex*, entraîne une modification importante dans le processus d'oviposition.

L'azadirachtine est rapidement dégradée dans l'environnement par les ultraviolets, la chaleur et l'alcalinité (Jarvis *et al.,* 1998). Sa durée de vie est donc limitée ce qui est à la fois un avantage du point de vue écologique (biodégradabilité) et un inconvénient du point vue de la synthèse. Effectivement, l'instabilité de la molécule et la complexité de sa structure, avec ses seize centres asymétriques, rendent le composé difficile à synthétiser. La synthèse totale de la molécule d'azadirachtine n'a d'ailleurs pas encore été réalisée. Plusieurs travaux traitant de la synthèse totale ou partielle de l'azadirachtine font d'ailleurs état de nombreux essais infructueux (Pflieger *et al.,* 1987; Nishikimi *et al.,* 1989; Ley, 1994; Watanabe *et al.,* 1996)

Il a toutefois été démontré (Isman, 1997), qu'indépendamment l'une de l'autre, la synthèse des deux portions, la portion décaline et la portion acétale hydroxyfurane, de la molécule d'azadirachtine (Figure 1.4), ne présentaient aucune activité IGR et très peu d'action antiappétante contrairement à la molécule entière d'azadirachtine.



Azadirachtine Portion décaline Portion hydroxyfurane

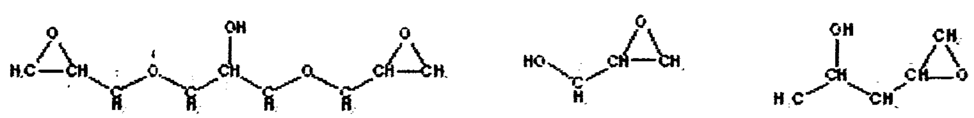
FIGURE 1.4 Portion décaline et portion acétale hydroxyfurane de la molécule d'azadirachtine.

Hypothèse et objectifs

C'est en fonction de ces derniers résultats que nous avons formulé l'hypothèse suivante. Nous croyons que la synthèse de molécules simples, ayant des similarités structurales avec le système époxy-alcool (constitué des carbones 1 à 4, voir Figure 1.4) de l'azadirachtine, qui correspond en fait à la jonction entre la portion décaline et la portion acétale hydroxyfurane, est une piste intéressante pour l'étude de composés stables du point vue moléculaire et possédant les caractéristiques insecticides de l'azadirachtine, à savoir sa toxicité et sa spécificité envers les espèces cibles. Aucune recherche jusqu'à présent ne s'est intéressée à la synthèse et l'étude entomopathogénique de structures simples dérivées du pont époxy-alcool de l'azadirachtine. L'étude de telles molécules possédant un pouvoir insecticide potentiel devient dès lors une voie prometteuse.

En fonction de l'hypothèse émise, les objectifs spécifiques formulés dans le cadre de cette recherche sont la synthèse de molécules simples similaires au système époxy-alcool de l'azadirachtine et l'étude entomopathogénique *in vivo* et *in vitro* des produits synthétisés.

Le premier objectif a été de cibler trois époxy alcools (Figure 1.5). Deux de ces molécules (I) 1,3-(bis (2,3-époxypropoxy)-propan-2-ol (aussi appelé en anglais glycerol diglycidyl ether) et (II) 2,3-époxy-propan-1-ol (aussi appelé glycidol) sont des époxy alcools disponibles commercialement dont la structure est similaire au système époxy alcool correspondant à l'alcool en position sept de l'azadirachtine. Pour la première molécule (I), il s'agit de la longueur de chaîne entre l'époxy et l'alcool avec un oxygène en plus. Pour la seconde molécule (II), on retrouve un carbone en moins dans la chaîne reliant l'époxy à l'alcool. Finalement la troisième molécule est le résultat de la synthèse par oxydation du 4-penten-2-ol avec l'acide méta-chloroperbenzoïque dans le dichlorométhane (Guan *et al.*, 2000; An *et al.*, 2001; Aurrecoechea *et al.*, 2001). La structure de cette dernière molécule (III) est très semblable en disposition et longueur de chaîne au système époxy alcool de l'azadirachtine.



(I) (II) (III)

FIGURE 1.5 Molécules époxy alcools utilisées pour l'étude entomopathogénique *in vivo* et in *vitro*. (I) 1,3-(bis (2,3-époxypropoxy)-propan-2-ol; (II) 2,3-époxy-propan-1-ol et (III) 4,5-époxy-pentan-2-ol.

Aussi en raison de la chiralité de la molécule (II) 2,3-époxy-propan-1-ol, les deux énantiomères R et S feront également l'objet de bioessais afin de déterminer si l'une ou l'autre des configurations domine. Bien que la molécule (I) 1,3-(bis (2,3-époxypropoxy)-propan-2-ol soit un mélange de plusieurs isomères et que la molécule (III) 4,5-époxy-pentan-2-ol soit un mélange de 4 produits (2 paires d'énantiomères) un manque de temps et de disponibilité des produits nous ont contraint à délaisser les testes avec ces stéréoisomères.

Le deuxième objectif consiste à vérifier l'activité insecticide des trois époxy alcools (I), (II) (III) (Figure 1.5) par des tests d'entomotoxicité sur des larves de lépidoptère *Galleria mellonella* L. et de comparer les résultats obtenus avec la toxicité de l'azadirachtine et celle d'un produit commercial à base d'huile de neem. Depuis l'extraction de l'azadirachtine à partir de la plante en 1968 (Butterworth et Morgan, 1968; 1971) l'azadirachtine a été l'objet de nombreux essais. Des essais en laboratoire ont indiqué que les extraits de neem étaient efficaces contre un vaste étendu d'espèces d'insectes (Koul, 1999). Plusieurs effets allant de l'antiappétant à des effets négatifs sur la reproduction ont aussi été largement rapportés. En comparant nos résultats avec la littérature existante nous serons donc en mesure d'évaluer l'entomotoxicité des époxy alcools avec celle de l'azadirachtine ou des extraits d'huile de neem. Cette partie de la recherche fait l'objet du premier article que l'on retrouve intégralement au chapitre suivant.

Finalement le dernier objectif, traitant la partie *in vitro* de l'étude, vise à comparer les effets cytotoxiques de l'azadirachtine avec les trois époxy alcools (I), (II) (III) (Figure 1.5) sur trois ordres insectes différents soit les coléoptères, les diptères et les lépidoptères. Les effets sur la croissance cellulaire sont faiblement documentés (Nisbet *et al.,* 2001). Toutefois des essais avec des coléoptères concluent que la mitose était affectée par l'azadirachtine (Schlüter, 1987). D'autres études ont démontré un accroissement de l'effet mitotique, soit la diminution de la division cellulaire, sur des cellules de lépidoptères traitées avec l'azadirachtine (Salehzadeh *et al.*, 2003). Nous pourrons ainsi quantifier et classifier, selon l'espèce testée, les valeurs calculées de concentrations létales (CL_{50}) de nos trois époxy-alcools ainsi que de l'azadirachtine. De plus, il nous sera possible de comparer les résultats de notre étude avec les articles publiés. Les résultats obtenus font l'objet du deuxième article qui se retrouve au dernier chapitre du présent mémoire.

MÉTHODOLOGIE

Pour les bioessais mesurant l'activité insecticide *in vivo*, des larves de *Galleria mellonella* L. ont été utilisées. Ce lépidoptère a été choisi en raison de sa sensibilité et des effets sur la croissance que provoquent les produits dérivés du neem (Schmutterer, 1990).

Nous avons procédé en appliquant 100 µL à diverses concentrations des différents produits à tester soit les trois époxys alcools; molécules I, II et III (Figure 1.5) ainsi que l'huile de neem commerciale et l'azadirachtine. Les produits ont été déposés par pipetage sur une diète standardisée. Un délai de 1h a ensuite été alloué avant l'ajout des larves, afin de permettre l'évaporation de l'acétone qui a été utilisée afin de dissoudre deux produits qui étaient insoluble en milieux aqueux (l'huile de neem ainsi que la molécule I). Dans le but de standardiser la méthode nous avons respecté le même délai pour la dilution des produits ne nécessitant pas d'acétone. Des larves, d'un poids standardisé, ont été individuellement placées en contact, dans des petits contenants ventilés, avec la nourriture. Les larves ont ensuite été remises dans les conditions d'élevage et observées chaque jour après l'application des produits. Deux contrôles ont également été réalisés, en raison des différents solvants de dilution utilisés pour l'expérience. Ces expériences nous ont permis d'ordonner, selon la concentration donnant 100% de mortalité, la toxicité des différents produits utilisés. Nous avons également noté la présence de déformations morphologiques chez les adultes émergents ayant été traités avec les produits.

Nous avons aussi réalisé une expérience d'inoculation d'une solution stérile d'azadirachtine par injection intrahemocoelique (Garzon *et al.*, 1990) sur douze larves. Nous avons procédé de la sorte suites aux essais d'applications d'azadirachtine pure sur diète, qui n'ont révélé aucune toxicité (% de mortalité nul). En raison des valeurs de concentration toxique d'azadirachtine rapportées dans la littérature, nous avons procédé par injection afin de couvrir une large plage de concentration allant de la dose sublétale aux applications beaucoup plus concentrées d'azadirachtine. L'injection intrahemocoelique nous a aussi permis de différencier entre la DL_{50}, c'est à dire la dose appliquée et la CL_{50}, la concentration réelle se retrouvant dans l'organisme de l'insecte et ainsi récolter un maximum d'informations. Les détails de la méthodologie concernant la partie *in vivo* sont décrits dans le premier article au chapitre II.

Pour le travail traitant la partie *in vitro* de l'étude, les mêmes produits que cités précédemment ont été utilisés soit les trois époxy alcools molécules I, II et III (Figure 1.5) ainsi que l'huile de neem commerciale et l'azadirachtine. Trois lignées cellulaires, provenant de trois importants ordres d'insectes, ont été utilisées lors des essais soit, le lépidoptère *Spodoptera frugiperda* J.E. Smith (Sf9), le diptère *Aedes triseriatus* Say (A.t. GRIP-1) et le coléoptère *Leptinotarsa decemlineata* Say (Ld-L1). Les essais ont été réalisés en trois parties; premièrement les tests cytotoxiques, deuxièmement le dénombrement cellulaire et troisièmement la coloration des chromosomes chacun visant à cumuler des informations différentes et complémentaires sur l'effets des produits appliqués.

Pour les tests cytotoxiques, nous avons procédé en inoculant, sur des microplaques de culture, les différentes lignées cellulaires (Sf9, A.t.GRIP-1 et Ld-L1). Un temps d'incubation de 24h à 28 °C a été alloué avant l'ajout des différents produits à diverses concentrations. Pour les mêmes raisons que les bioessais, deux contrôles ont également été réalisés. Après une période post-traitement de 24h et 7 jours aux mêmes conditions d'inoculation, les cellules ont été observées sous microscope. Cette partie de l'étude nous a permis de déterminer la concentration limite des différents produits ayant un effet sur les cellules. Ce test nous a été utile afin de relever les caractéristiques morphologiques des cellules ayant subies des dommages.

Dans cet essai, nous avons également soumis, les mêmes lignées cellulaires ayant préalablement été stimulées avec l'hormone juvénile et l'insuline aux mêmes tests. Ces deux hormones, l'hormone juvénile et l'insuline sont, ou dans le cas de l'insuline fait office, d'hormone de croissance chez l'insecte. En raison de la littérature traitant des sites possibles d'action de l'azadirachtine au niveau cellulaire (Schlüter, 1987; Nisbet *et al.,* 2001; Salehzadeh *et al.,* 2003) nous voulions vérifier si une stimulation avec ces deux hormones de croissance pouvait avoir un effet et ainsi modifier l'action des produits, sur la concentration limite ayant des effets sur les différentes cellules.

Pour le dénombrement cellulaire, nous avons procédé exactement comme pour les tests cytotoxiques, mais en ayant préalablement soin de dénombrer, en utilisant un haemocymètre, le nombre de cellules initiales inoculées dans chaque boite de culture. Après incubation des différentes lignées cellulaires, aux mêmes conditions citées précédemment, les produits ont été ajoutés aux concentrations ayant un effet sur cellules déterminées par les tests cytotoxiques précédents. Après une période post-traitement de 48h et 7 jours, les cellules ont de nouveau été dénombrées. Cet essai nous a permis d'établir le taux de croissance (ou de décroissance) des cellules ayant été traitées et ainsi quantifier l'effet des produits sur la division cellulaire.

La dernière technique utilisée, la coloration des chromosomes, se résume en trois étapes selon la méthode décrite par Charpentier et al. (2002). Dans un premier temps, un culot de cellules ayant subit les effets des différents produits est lavé puis fixé dans un mélange d'acide acétique et de méthanol. Ensuite vient la coloration à l'orcéine. Finalement, entre deux lames de microscope une goutte de suspension cellulaire colorée est pressée afin de libérer les chromosomes. Sous microscope, les cellules présentant une métaphase sont dénombrées. Cette technique nous permet ainsi de quantifier l'effet des différents produits appliqués sur la mitose cellulaire (arrêt de la division cellulaire) des trois lignées cellulaires. Les techniques complètes ainsi qu'une description détaillée des conditions expérimentales figurent dans le dernier chapitre.

RÉSULTATS

Étant donné que les résultats recueillis au cours de cette recherche font l'objet de deux articles soumis, présentés respectivement aux chapitre II et III du présent mémoire, je ne citerai, dans cette section, que les grandes lignes des résultats obtenus tant pour la partie *in vivo* que pour la partie *in vitro* de l'étude. Également je ferai mention de résultats complémentaires n'ayant pas été rapportés dans les deux articles.

Notre étude révèle que les trois molécules époxy-alcools; (I) 1,3-(bis (2,3-époxypropoxy)-propan-2-ol); (II) 2,3-époxy-propan-1-ol et (III) 4,5-époxy-pentan-2-ol (Figure 1.5) sont plus toxiques pour les larves de *Galleria mellonella* que l'huile de neem commerciale et davantage encore que l'azadirachtine qui n'a d'effet que par injection. Pour tous les produits, on voit clairement au tableau suivant (Tableau 1.1) une relation dose dépendante entre le pourcentage de larves mortes et la concentration des produits appliqués.

À partir des concentrations de produits appliquées donnant un pourcentage de mortalité de 100%, un ordre de toxicité des produits a pu être établi (Figure 1.6). Dans l'ordre décroissant de toxicité il y a le 2,3-époxy-propan-1-ol, 4,5-époxy-pentan-2-ol et le 1,3-(bis (2,3-époxypropoxy)-propan-2-ol). Cet ordre de toxicité a d'ailleurs été corroboré par l'estimation des DL_{50}.

Les essais voulant différencier entre les deux configurations de la molécule 2,3-époxy-propan-1-ol ont révélé qu'aucun des deux énantiomères R et S ne domine En fait les deux produits sont aussi toxiques l'un que l'autre. Des applications de 100 μL (0.12mg/g) de glycidol R et S ont révélées des toxicités comparables avec des pourcentages de mortalité respectivement de 87% et

100% comparativement au témoin où seulement 13 % de mortalité ont été observés.

Concentration du produit appliqué (mg/g)	Larves mortes (%)
Contrôle	21
Contrôle acétone	20
Application 1,3-(bis (2,3-époxypropoxy)-propan-2-ol	
0.40	100
0.20	60
0.04	20
0.004	3
Application 2,3-époxy-propan-1-ol	
0.30	100
0.15	100
0.12	100
0.06	80
0.03	77
0.015	40
0.010	20
Application 4,5-époxy-pentam-2-ol	
0.45	100
0.23	100
0.10	70
0.05	40
Application d'azadirachtine	
0.20	17
0.004 injection	100
Application huile de neem Nature™	
70	100
28	60
14	60
2.8	30

TABLEAU 1.1 Taux de mortalité (%) chez les larves de *Galleria mellonella* en fonction des différentes applications de produits. Au tableau suivant sont exposées les relations dose dépendance entre le pourcentage de larves mortes et la concentration des trois molécules époxy alcool, de l'azadirachtine et d'extrait d'huile commerciale à base de neem.

Il apparaîtrait toutefois, une différence dans le temps d'action du produit. En effet, l'énantiomères R présente un temps d'action (i.e nombre jour pour la mortalité) moyen de 5,7 jours comparativement à 2,3 jours en moyenne pour l'énantiomère S. Tout comme pour les larves traitées avec le mélange d'énantiomères, les insectes traités avec le glycidol R et S présentaient des taches noires caractéristiques. Ces résultats complémentaires ne figurent pas dans les deux articles.

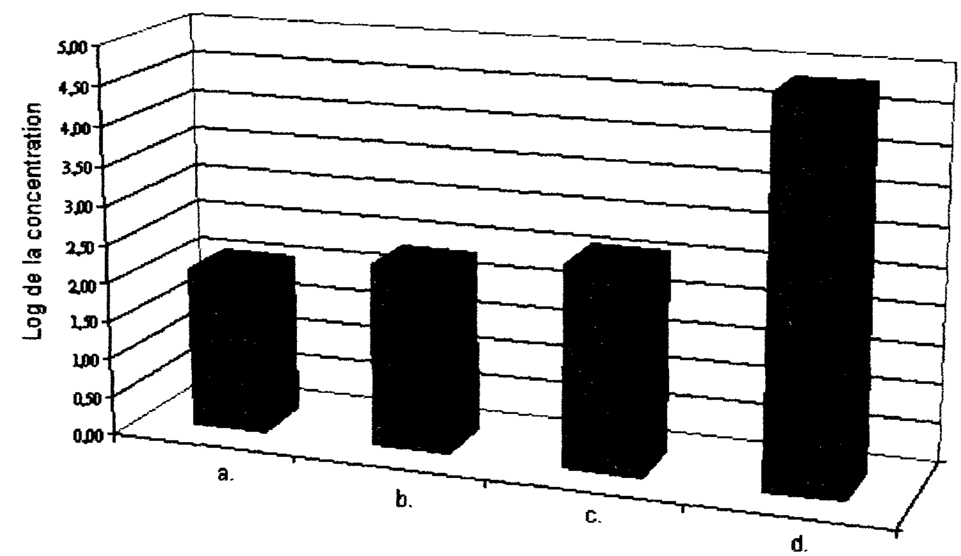


FIGURE 1.6. Logarithme de la concentration (mg/g) minimum de produit appliqué induisant 100% de mortalité chez les larves de *Galleria mellonella*. Dans le graphique ci haut, sont identifié par les lettres les produits suivants; a. 2,3-époxy-propan-1-ol, b. 4,5-époxy-pentan-2-ol, c. 1,3-(bis (2,3-époxypropoxy)-propan-2-ol) et d. l'huile de neem.

Outre les pourcentages de mortalité, des caractéristiques communes à plusieurs produits ont été observées. Comme rapporté par certains auteurs pour les produits à base de neem et l'azadirachtine (Gerard et Ruf, 1995; Mulla et Su, 1999), nous avons constaté l'effet répulsif pour tous les produits appliqués et notamment pour le 1,3-(bis (2,3-époxypropoxy)-propan-2-ol) pour lesquels les larves fuyaient la source du produit. Nous croyons que l'aspect visqueux du 1,3-

(bis (2,3-époxypropoxy)-propan-2-ol) pourrait expliquer ce comportement. Autre fait intéressant, plusieurs larves mortes présentaient quasi systématiquement des taches noires dans la région des fausses-pattes. Nous croyons que cet effet est dû à un phénomène de mélanisation résultant du contact toxique avec le produit. Une manifestation semblable chez des *Epilachna vaivestis* traités avec des extraits de neem a également été rapportée par Schmutterer (1990). Aussi, chez les adultes ayant émergé, certaines malformations morphologiques, surtout au niveau des ailes et des pattes, ont été observées pour tous les produits utilisés. Ces observations corroborent les faits rapportés par Schmutterer (1990) et Schlüter (1987) voulant que des malformations au niveau des ailes soit spécifiquement dû à un effet du neem sur le bourgeon de l'aile.

À la lumières des résultats concluants obtenus sur larves d'insectes, nous avons voulu investiguer davantage les effets de nos produits en travaillant au niveau cellulaire. Il est connu que l'azadirachtine induit la mort chez l'insecte notamment à cause de son action inhibitrice sur la croissance (Lowery et Isman, 1994; Mordue *et al.,* 1998; Manal et Sehnal, 2000), Comme des effets comparables ont été observés lors de notre étude *in vivo*, à savoir, des mues incomplètes ou encore des déformations morphologiques voire même la mort de l'insecte comme rapportée précédemment, une étude cytotoxique de nos produits s'imposait d'elle-même.

Nos résultats démontrent clairement un effet cytotoxique immédiat et retardé de tous les produits utilisés dans cette étude. Les trois lignées cellulaires Sf9, A.t. GRIP-1 et Ld-L1 présentent une sensibilité similaire pour chaque produit individuel. Dans cette étude un ordre de toxicité des produits, moins évident que celui de l'étude *in vivo*, a pu être établi. Les cellules étaient davantage affectées lors de l'application de l'azadirachtine (0.7 à 7.2 ppm) suivit du 1,3-(bis (2,3-époxypropoxy)-propan-2-ol) (307 à 410 ppm) et de l'huile de neem commercial (418 à 558 ppm) et finalement du 2,3-époxy-propan-1-ol (1393 à 2230 ppm) et du 4,5-époxy-pentan-2-ol (1700 à 2126 ppm). En raison de leurs effets cytotoxiques, cette classification des produits diffère quelque peu de celle établie pour les larves de *Galleria mellonella.* Nous croyons que des mécanismes comme la détoxification chez l'insecte ou encore la complexité du mécanisme d'action des produits dans un organisme complexe peut expliquer ces différences. Contrairement à ce que l'on aurait pu croire, le prétraitement aux hormones n'a eu aucun effet sur la toxicité des produits appliqués. Selon Schlüter (1987), la complexité du système endocrinien des insectes holométaboles, i.e. à métamorphose complète avec un stade nymphal, par opposition aux insectes hétérométaboles chez qui le passage de l'état larvaire à l'état adulte se fait directement, expliquerait l'absence de la diminution de l'effet toxique de l'azadirachtine recherchée par Schlüter suite aux traitements aux hormones. Ces mêmes études ont toutefois démontré au contraire une diminution de l'effet de l'azadirachtine chez des insectes hétérométaboles traités aux hormones. Comme les trois lignées de cellules utilisées proviennent d'insectes de type holométabole, il semble que le système endocrinien complexe ait également contré l'effet d'amplification de toxicité de l'azadirachtine recherché suite à nos traitements hormonaux. Par conséquent les traitements aux hormones ont été sans effet sur la toxicité de l'azadirachtine dans notre cas.

Comme pour les larves de lépidoptères, des déformations ont été observées sur les cellules. De façon générale, les cellules s'arrondissaient ou même gonflaient; ce phénomène a d'ailleurs été observé par Reed et Majumdar (1998). Dans certains cas, des vacuoles dans le cytoplasme (un mécanisme de défense chez la cellule), ont été observées. Cette réaction de la cellule a d'ailleurs déjà été rapportée par Cohen et al. (1996). D'autres effets cytotoxiques comme la formation d'agrégats ou plus évidents encore, la lyse induisant ainsi la mort cellulaire sont aussi parmi les observations.

Le dénombrement cellulaire a permis de quantifier l'effet cytotoxique des produits. La figure 1.7 révèle très bien l'effet post traitement sur la croissance cellulaire des trois lignées comparativement au contrôle. Comme rapporté par Salehzadeh et al. (2002), il apparaît que la lignée cellulaire la plus sensible au traitement de l'azadirachtine soit la *Spodoptera frugiperda* J.E. Smith (Sf9). Toutefois cette même lignée a vu sont accroissement cellulaire augmenté lors du traitement avec le 4,5-époxy-pentan-2-ol contrairement aux deux autres lignées.

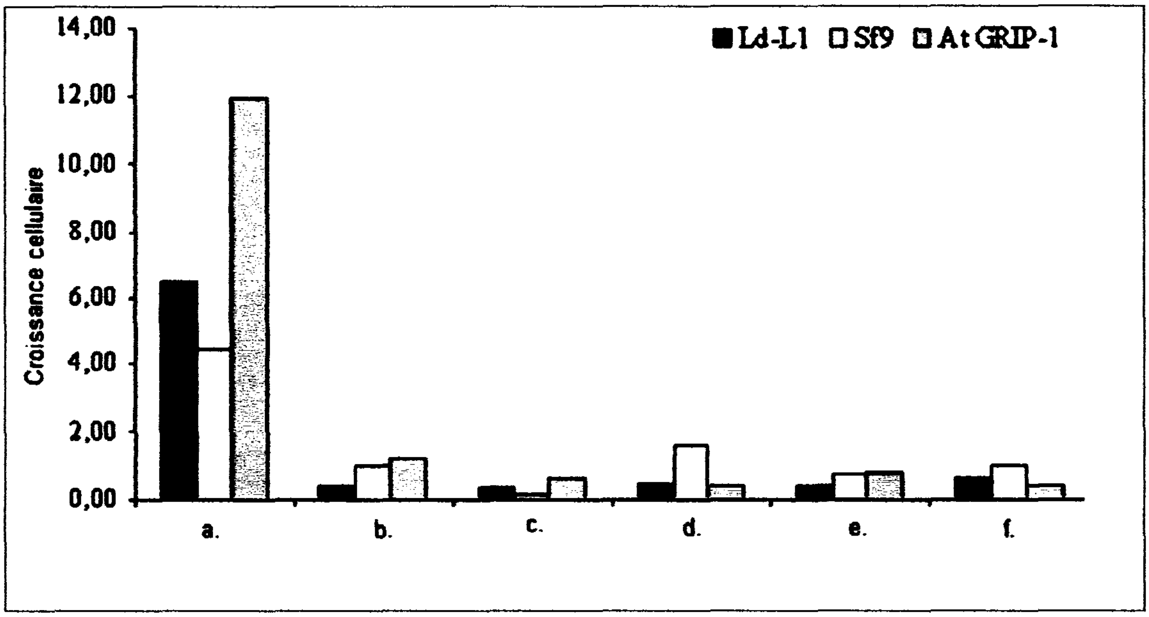


FIGURE 1.7 Effet des différents produits après 7 jours de contact sur la croissance cellulaire des lignées Ld-L1, Sf9 et A.t. GRIP-1. La croissance cellulaire est exprimée en ratio du nombre de cellules / mL au temps 7 jours divisé par le nombre de cellules / mL au temps 0. Sont identifié par les lettres les produits suivants; a. contrôle, b. contrôle acétone, c. azadirachtine, d. 4,5-époxy-pentan-2-ol, e. 2,3-époxy-propan-1-ol et f. 1,3-(bis (2,3-époxypropoxy)-propan-2-ol).

Finalement la dernière partie de cette étude corrobore les résultats précédant en imageant l'effet mitotique des produits sur les cellules. L'arrêt de la division cellulaire, la mitose, était significativement plus élevé chez les cellules de Ld-L1 ayant subit un traitement comparativement aux témoins. Contrairement au Ld-

L1, chez les cellules A.t. GRIP-1 le phénomène n'a pu être démontré. Quant aux cellules de Sf9, la mitose a été observée lors des applications d'azadirachtine seulement

CONCLUSION

Cette étude nous permet de conclure que nos molécules époxy alcool présentent une cytotoxicité sur culture de cellules similaire aux effets toxiques observés *in vivo* sur larve de *Galleria.* Ces derniers résultats sont aussi comparables à ceux rapportés dans la littérature pour l'azadirachtine et les produits à base de neem. Nous ne pouvons affirmer si nos molécules ont le même mode d'action que l'azadirachtine, toutefois nos trois molécules présentent les deux fonctionnalités, soit le groupement alcool et le groupement époxy, similaire au pont entre les deux moitiés de l'azadirachtine tel que décrit dans notre hypothèse. De ce fait nous pouvons supposer que ces deux fonctionnalités disposées de façon similaire à celles retrouvées dans la molécule d'azadirachtine peuvent présenter les avantages du bioinsecticide soit son pouvoir insecticide et sa sélectivité.

Bien que nombre d'informations aient été cumulées lors de cette recherche, les résultats obtenus ouvrent la porte à une étendue de possibilités pour de futurs travaux. Par exemple, il serait intéressant de déterminer la contribution de l'effet toxique de chacune des fonctionnalités, soit l'époxy et l'alcool, indépendamment l'une de l'autre. Aussi la synthèse de molécules époxy alcool ramifiées correspondant davantage à la structure plus complexe de l'azadirachtine serait une avenue intéressante pour l'approfondissement des connaissances. Une étude du mode d'action de nos molécules époxy alcool simples pourrait éventuellement permettre de comprendre le mode d'action plus complexe de l'azadirachtine. En effet en utilisant des techniques de marquage de nos molécules il serait ainsi possible de localiser les organes cibles dans l'insecte ou encore d'identifier les sites récepteurs de l'azadirachtine au niveau cellulaire.

BIBLIOGRAPHIE

An, M., Toochinda, T., Bartlett, P.A. (2001) "Five-membered ring analogues of shikimic acid", Journal of Organic Chemistry 66: 1326-1333.

Aurrecoechea, J.M., Pérez, E., Solay, M. (2001) "Synthesis of trisubstituted furans from epoxypropargyl esters by sequential SmI_2-promoted reduction-elimination and Pd(II)-catalyzed cycloisomerization", Journal of Organic Chemistry 66: 564-569.

Bliefert, C., Perraud, R. (2001) "Chimie de l'Environnement Air, Eau, Sols et Déchets", De Boeck Université, Paris, pp. 364-368.

Bourassa, J.P. (2000) "Le Moustique: par Solidarité Écologique", Les éditions du Boréal, Montréal, Québec, pp. 239.

Broughton, H.B., Ley, S.V., Slawin, A.M.Z., Williams, D.J., Morgan, E.D., (1986) "X-ray crystallographic structure determination of detigloyldihydroazadirachtin and reassignment of the structure of the limonoid insect antifeedant azadirachtin", Journal of Chemical Society 1: 46-47.

Butterworth, J.H., Morgan, E.D. (1968) "Isolation of a substance that suppresses feeding in locusts", Journal of Chemical Society. Chemical Communications 1: 23-24.

Butterworth, J.H., Morgan, E.D. (1971) "Investigation of the locust feeding inhibition of the seeds of the neem tree, *Azadirachta indica*", Journal of Insect Physiology 17: 969-977.

Charpentier, G., Tian, L., Cossette, J., Léry, X., Belloncik, S. (2002) "Characterization of cell lines developed from the Colorado potato beetle, *Leptinotarsa Decemlineata Say* (Coleoptera: Chrysomelidae)", In Vitro Cellular & Developmental biology-Animal 38: 73-78.

Cohen, E., Quistab, G.B., Casida, J.E. (1996) "Cytotoxicity of nimbolide, epoxyazadiracdione and other limonoids from neem insecticide", Life Sciences 58: 1075-1081.

Darazy-Choubaya, D. (2002) "La perception gustative des phytoecdystéroïdes par les larves de la pyrale du maïs, *Ostrinia nubilalis* Hübner (Lepidoptera, Pyralidae)", Thèse de doctorat, Institut national de recherche agronomique (INRA) Paris-Grignon.

Dev, S., Koul, O. (1997) "Insecticides of Natural Origin", Harwood Academic Publishers, Newark, USA, pp. 365.

Doucet-Leduc, H. (1991) "La contamination des aliments: une préoccupation d'aujourd'hui", Modulo éditeur, Ville Mont-Royal, pp. 248.

Garzon, S., Strykowski, H., Charpentier, G. (1990) "Implication of mitochondria in the replication of Nodamura virus in larvae of the Lepidoptera, *Galleria mellonella* (L.) and in suckling mice", Archives of Virology 113: 165-176.

Gerard, P.J., Ruf, L.D. (1995) "Effect of a neem (*Azadirachta indica* A. Juss, Meliaceae) extract on survival and feeding of larvae of four keratinophagous insects", Journal of Stored Products Research 31: 111-116.

Govindachari, T.R., Narasimhan, N.S., Suresh, G., Partho, P.D., Gopalakrishnan, G. (1996) "Insect antifeedant and growth-regulating activities of salannin and other c-seco lomonoïds from neem oil in relation to azadirachtin", Journal of Chemical Ecology 8: 1453-1461.

Guan, H.P., Ksebati, M.B., Kern, E.R., Zemlicka, J. (2000) "Approaches to unsaturated analogues of nucleosides comprising four- and six-membered rings", Journal of Organic Chemistry 65: 5177-5184.

Isman, M.B. (1997) "Neem insecticide", Pesticide Outlook 8: 32-38.

Jarvis, A.P., Johnson, S., Morgan, E.D. (1998) "Stability of the natural insecticide azadirachtin in aqueous and organic solvants", Pesticide Science 53: 217-222.

Koul, O. (1999) "Insect growth regulating and antifeedant effects of neem extracts and azadirachtin on two aphid species of ornamental plants", Journal of Biosciences 1: 85-90.

Kraus, W., Bokel, M., Cramer, R., Klenk, A., Pöehnl, H. (1985) "Constituents of neem and related species. A revised structure of azadirachtin", 3rd Int. Conf. Chem. Biotechnol. Biol. Act. Nat. Prod. 4: 446-450.

Larson, R.O. (1989) "The commercialization of neem" *dans* The neem tree, Focus on phytochemical pesticides, Vol. 1, Jacobson, M., CRC Press, Boca Raton.

Ley, S.V. (1994) "Synthesis and chemistry of the insect antifeedant azadirachtin", Pure and Applied Chemistry 66: 2099-2102.

Lowery, D.T., Isman, M.B. (1994) "Insect growth regulating effects of neem extract and azadirachtin on aphids", Entomologia Experimentalis et Applicata 72: 77-84.

Manal, M.A., Sehnal, F. (2000) "Azadirachtin potentiates the action of ecdysteroid agonist RH-2485 in *Spodoptera littoralis*", Journal of Insect Physiology 46: 267-274.

Ministère de l'Environnement. (1998) "Code de gestion des pesticides", Québec, bibliothèque nationale, Éditeur officiel du Québec, pp. 26.

Moreira, M.F., Garcia, E.S., Masuda H. (1994) "Inhibition by azadirachtin of phospholipid transfer from lipophorin to the Oocytes in *Rhodnius proxilus*", Archives of Insect Biochemistry and Physiology 27: 287-299.

Mordue, A.J., Simmonds, M.S.J., Ley, S.V., Blaney, W.M., Mordue, W., Nasiruddin, M., Nisbet, A.J. (1998) "Actions of azadirachtin, a plant allelochemical, against insects", Pesticide Science 54: 277-284.

Mulla, M.S., Su, T. (1999) "Activity and biological effects of neem products against arthropods of medical and veterinary importance", Journal of the American Mosquito Control Association 15: 133-152.

Nisbet, A.J., Mordue, A.J., Grossman, R.B., Jennens, L., Ley, S.V., Mordue, W. (2001) "Characterization of azadirachtin binding to Sf9 nuclei in vitro", Archives of Insect Biochemistry and Physiology 46: 78-86.

Nishikimi, Y., Iimori, T., Sodeoka, M., Shibasaki, M. (1989) "Synthetic studies of azadirachtin. Synthesis of the cyclic acetal intermediate in the naturally occurring form", Journal of Organic Chemistry 54: 3354-3359.

Painchaud, J. (1999) "La production porcine et la culture du maïs. Impacts potentiels sur la qualité de l'eau", Le Naturaliste Canadien, 123: 41-46.

Pflieger, D., Muckensturm, B., Robert, P.C., Simonis, M.T., Kienlen, J.C. (1987) "Synthesis of the furo-pyran moiety of the azadirachtin", Tetrahedron Letters 28: 1519-1522.

Reed, E., Majumdar, S.K. (1998) "Differential cytotoxic effects of azadirachtin on *Spodoptera frugiperda* and mouse cultured cells", Entomologia Experimentalis et Applicata 89: 215-221.

Riba, G., Silvy, C. (1989) Combattre les ravageurs des cultures enjeux et perspectives", INRA, Paris, pp. 230.

Salehzadeh, A., Akhkha, A., Cushley, W., Adams, R.L.P., Kusel, J.R., Strang, R.H.C. (2003) "The antimitotic effect of the neem terpenoid azadirachtin on cultured insect cells", Insect Biochemistry and Molecular Biology 33: 681-689.

Salehzadeh, A., Jabbar, A., Jennens, L., Ley, S.V., Annadurai, R.S., Adams, R., Strang, R.H.C. (2002) "The effects of phytochemical pesticides on the growth of cultured invertebrate and vertebrate cells", Pest Management Science 58: 268-276.

Schlüter, U. (1987) "Effects of azadirachtin on developing tissues of various insect larvae" Dans "Natural pesticides from the neem tree (*Azadirachta indica* A. Juss) and other tropical plants", Proceedings of the Third International Neem Conference, Nairobi, Kenya, 10-15 july 1986 (ed. by Schumutterer H & Ascher KRS). Deutsche Gesellschaft für Technische Zusammenarbeit (GTZ) GmbH. pp. 331-348.

Schmutterer, H. (1990) "Properties and potential of natural pesticides from the neem tree, *Azadirachta indica*", Annual Review of Entomology 35: 271-297.

Schmutterer, H., Ascher, K.R.S. (1987) "Natural pesticides from the neem tree (*Azadirachta indica* A. Juss) and other tropical plants", Proceedings of the Third International Neem Conference, Nairobi, Kenya, 10-15 july 1986 (ed. by Schumutterer H & Ascher KRS). Deutsche Gesellschaft für Technische Zusammenarbeit (GTZ) GmbH 703 pages.

Scott, I.M., Kaushik, N.K. (1998) "The toxicity of the Margosan-O, a product of neem seeds, to selected target and non target aquatic invertebrates", Archives of Environmental Contamination and Toxicology 35: 426-431.

Su, T., Mulla, M. (1999) "Oviposition bioassay responses of *Culex tarsalis* and *Culex quinquefasciatus* to neem products containing azadirachtin", Entomologia Experimentalis et Applicata 91: 337-345.

Tron, I., Piquet, O., Cohuet, S. (2001) "Effets chroniques des pesticides sur la santé : état actuel des connaissances", observatoire régional de la santé en Bretagne, Bretagne, 87 pages.

Vincent, C., Coderre, D. (1992) "La lutte biologique", Gaëtan Morin ,Québec, 671 pages.

Ware, G.W. (2000) "The Pesticide Book", 5e éd.. Thomson Publication, Arizona, 415 pages.

Watanabe, H., Watanabe, T., Mori, K. (1996) "Enantioselective synthesis of the tricyclic furan moiety of azadirachtin, a potent insect antifeedant", Tetrahedron 52: 13939-13950.

CHAPITRE II

PREMIER ARTICLE

Effects of azadirachtin and of simpler epoxy-alcohols on survival and behaviour of *Galleria mellonella* (Lepidoptera) larvae.

Carole Charbonneau,

Roland Côté

Guy Charpentier

Département de Chimie-Biologie, Université du Québec à Trois-Rivières, Trois-Rivières, Québec, Canada, G9A 5H7.

Short title: Effects of azadirachtin and derivatives on *Galleria mellonella*.

Keywords: Bioinsecticide; Toxicity; Azadirachtin; Neem; Lepidoptera.

Abstract

Investigation of the toxicity of three molecules based on the epoxy-alcohol fragment of azadirachtin: glycerol diglycidyl ether, glycidol (GDE), 4,5-epoxy-pentan-2-ol, azadirachtin and neem extracts have revealed insecticidal activity on Lepidoptera *Galleria mellonella* L. larvae. These epoxy-alcohols were more toxic for *G. mellonella* larvae than the a commercial neem product and much more active than the azadirachtin that killed the larvae only after injection. The epoxy-alcohol doses giving 100% mortality were in the increasing order: glycidol, 4,5-epoxy-pentan-2-ol, and, finally, GDE. Our results confirm the importance of the epoxy-alcohol junction between the two parts of the azadirachtin molecule for biological activity. Other effects of the epoxy-alcohols tested were blackening of larvae, death as pupae and similar morphological deformities of adults as described in the literature for azadirachtin or neem extracts.

Introduction

Since the discovery of the DDT, an organochlorine pesticide, products for pest management have been a threat to human health and to the environment, causing undesirable effects like pollution, health problems, development of insecticide resistance, or negative effects on nontarget organisms. One alternative to chemical insecticides is the use of plant extracts. Azadirachtin (I) (Figure 2.1), a plant-extracted compound, is of growing interest for pest control research as well as commercial selling (Ley *et al.* 1993a). Azadirachtin was extracted from the neem tree *Azadirachta indica* (A. Juss), a plant from the Meliaceae family, by Butterworth and Morgan (1968, 1971) and identified as a highly oxygenated tetranorterpenoid by Kraus *et al.* (1985) and Broughton *et al.* (1986). This substance gave rise to a great interest because of its insecticidal activity and biodegradability. Laboratory testing indicates that neem extract-based insecticides are effective against more than 400 pest species (Koul 1999). Azadirachtin presents no persistence in the environment because this compound is rapidly degraded by UV light (photosensitive), humidity, high temperature and mildly alkaline solution (Jarvis *et al.* 1998). Neem products are also relatively safe to mammals and humans (Schmutterer 1990). Unlike many chemical insecticides that are specific in their actions against pests, azadirachtin produces strong anti-insect effects (Schmutterer 1990; Mordue *et al.* 1998; Su and Mulla 1999) including repellent effects, feeding deterrence (antifeedancy) and growth regulation caused mainly by alterations of ecdysteroid and juvenile hormone concentration. Some effects can also occur during metamorphosis, such as impaired wing development (Schlüter 1987). Negative effects on reproduction have also been reported, such as low oviposition rates (Medina *et al.* 2004; Mulla and Su 1999), egg viability reduction (Bruce *et al.* 2004); more clearly, an ovicide effect (Su and Mulla 1998) has been also observed.

Azadirachtin, with its complex array of oxygen functionality, stereogenic and quaternary centres and chemical instability, provides a challenging compound for further studies. Several works have focused on its total or partial synthesis (Pflieger *et al.* 1987; Nishikimi *et al.* 1989; Watanabe *et al.* 1996). Attempts to synthesize the whole molecule have remained unsuccessful for over ten years (Ley 1994; Ley *et al.* 1993b, 1991). While the two major portions of the molecule, the decalin portion and the hydroxyfuran acetal moiety, have been synthesized, neither has any insect growth regulatory (IGR) activity and only modest levels of antifeedant action (Isman 1997). We have become interested in the link between those two portions (Figure 2.2) because the study of the linkage between these two moieties seems to be a fundamental target area to study. In order to explore this portion of the azadirachtin molecule, we decide to investigate simpler molecules based on epoxy-alcohols, a template mimicking this crucial bond between the two azadirachtin fragments (Figure 2.1). The aim of the present work is to compare the structure–activity relationships of azadirachtin with simpler analogues related to fragment (C_2-C_4) of the natural product. We hope to find a more stable and simpler structure, with biological activity similar to that of azadirachtin.

Material and Methods

Chemicals

Glycerol diglycidyl ether (GDE) (1,3-(bis (2,3-epoxypropoxy)-propan-2-ol) tech. grade (II) (Figure 2.1) was purchased from Aldrich Co. (Oakville, Canada).

Glycidol (2,3-epoxy-propan-1-ol) purity 96% (III) (Figure 2.1) was purchased from Aldrich Co. (Oakville, Canada).

The synthetic epoxy alcohol, 4,5-epoxy-pentan-2-ol (IV) (Figure 2.1), was prepared by the oxidation the 4-penten-2-ol with meta-chloroperbenzoic acid in dichloromethane (Figure 2.3) as described by An *et al.* (2001), Aurrecoechea *et al.* (2001) and Guan *et al.* (2000). The synthetic product was purified by silica gel column chromatography and characterized using infrared, ^{13}C and ^{1}H nuclear magnetic resonance spectroscopy. The observed peak position, multiplicity and intensity are consistent with the calculated values for the proposed molecule.

The neem formulation was Nature's neem oil™ from Grotek Manufacturing Inc. (BC, Canada) and contained 2500 ppm of the active ingredient azadirachtin.

The 95% pure azadirachtin (I) (Figure 2.1) was purchased from Sigma-Aldrich Canada Ltd (Oakville, Canada).

Insects

The Lepidoptera species used in our tests was *Galleria mellonella* L. Larvae were obtained from the Faculté des Sciences et Techniques, Université de Haute Normandie, (Rouen, France) and were continuously reared under controlled environmental conditions (28 ± 2°C; 50 ± 5% relative humidity; 0:24 light: dark). They were fed a semiartificial diet composed of honey, glycerol, mixed cereals (Pablum®) and yeast. This insect was chosen because the Lepidoptera belong to the most sensitive groups of insects with regard to growth effects of neem (Schmutterer 1990).

Toxicity and insecticidal activity assays

For the bioassays, 100 µL of GDE was dissolved in pure acetone at different doses (from 0.40 to 0.004 mg/g of diet) were added to the weighed diet (0.35 ± 0.05 g). Also 100 µl of Glycidol diluted in non-demineralized distilled water at different doses from 0.30 to 0.010 mg/g was added to the same amount of diet. The 4,5-epoxy-pentan-2-ol was applied directly on diet at different doses from 0.45 to 0.05 mg/g. The neem oil formulation at different doses was also added to the weighed diet either directly (from 420 to 70 mg/g) or diluted in 40% acetone aqueous solution for lower doses (28 to 2.8 mg/g). Finally, each flask containing 0.5 mg of pure azadirachtin was dissolved in 100 µl of acetone and diluted in non-demineralized distilled water for application to the weighed diet at different doses (from 0.20 to 0.0002 mg/g).

The treated diets were left to dry 1 hour at room temperature before the larvae were added. Larvae of 0.15 ±0.05 g standardized weight were placed into individually labelled 30 ml plastic cups (Jet Plastica Industries Inc., Hatfield, USA) with well vented caps (James River Corporation, Norwalk, USA). We also performed an experiment with twelve larvae injected by intrahemocoelic inoculation of a sterile solution of pure azadirachtin at 4 x 10^{-3} mg per g of larvae body weight (Garzon *et al.* 1990). The treated larvae were maintained in the same environmental conditions as above. Two controls were done, one diet-treated with 100 μl of aqueous solution of acetone 40 % and the other with 100 μl of non-demineralized distilled water. Every day after the application of chemicals the molt and the mortality of treated and control larvae were monitored. The experiments were set up in three replicates, each with 10 larvae with standardized weight per replicate, so that 30 larvae were tested for each dose of each chemical.

Statistical analysis

To obtain the LD_{50} (lethal doses 50%), the data were subjected to probit analysis (Finney 1971).

Results

Assays with glycerol diglycidyl ether

The larvae death was dose-dependent (Table 2.1). High dose of GDE, (0.40 mg/ g of diet), killed all larvae before they molted in to adult. The application of product induced an immediate inhibition of silk production and no cocoon was observed. About 30% of the dead larvae treated with GDE at 0.40 mg/g presented some black spots on the stomach specifically on the proleg area. Larvae fed with 0.20 mg to 0,004 mg of GDE per gram of diet, metamorphosis in adult occurred at similar intervals, the adult insect leaving the pupal case after about 18 days. Larvae fed with 0.20 mg/g GDE died in proportion of 60%. Half of them died as larvae and the remainder perished as pupae. Black spots were also observed around the proleg area in 22% of the dead larvae. About 8% adult insects showed malformations (Figure 2.4 b). The left wing of a specimen was atrophied compared with the reference butterflies (Figure 2.4 a). In the group of larvae fed with 0.04 mg/g GDE, mortality was about 20%. In comparison with the high doses, more deaths occurred during the pupal stage (83% of the death). Again, dead larvae presented dark spots near proleg. Only one insect presented morphological deformities, this specimen being unable to exit its silken case. The lower dose of glycerol diglycidyl ether produced a negligible mortality. Test with GDE at 0,004 mg/g of diet indicated that this dose was inactive and allow an apparently normal larval development. Population emerged in proportion of 97%, that is almost 20% over the viable adult emergence rate obtained with the acetone control. The remainder, 3% of larvae, died in 16 days.

Assays with glycidol

Glycidol induced a rapid death in *G. mellonella* when a dose of 0.30 mg/g was added to the larvae diet (Table 2.1). All the larvae died within 24 hours. More than a half of dead larvae were completely black from head to the proleg area, in comparison with the control were the dead larvae were totally grey. Like in the assays with GDE, an inhibition of silk production was observed. Feeding larvae with 0.15 mg/g of glycidol caused 100% mortality (Table 2.1); 83% died within 24 hours and the other 17% have a mean length of time of 16 days before death. As in the higher dose, they were half black. In comparison with the control, the cocoons formed were smaller and fragile. A dose of 0.12 mg/g of glycidol killed the totality of treated larvae (Table 2.1) with 63% of larvae dying within 24 hours, another 30% in about two weeks and 7% died at the prepupal stage. Again almost all of the dead larvae were partly black. Larvae fed with 0.06 mg/g of glycidol died in a proportion of 80% (Table 2.1). Compared with a dose five time higher dose (0.30 mg/g) this dose extended the time before death from 24 hours to 6 days for 80% of larvae, the rest dying during the pupal stage. The over all dead larvae growth was reduced, about 60% of the control value. Only 20% of larvae emerged as adult and 33% of them accomplished an unsuccessful metamorphosis (Figure 2.4 d). One specimen treated with a dose of 0.06 mg/g dose of glycidol presented physical abnormalities; both wings were atrophied and had deformities at legs. Death before adult emergence (77%) occurred when the larvae were fed with 0.03 mg/g of glycidol (Table 2.1). Some insects died as prepupae (35%), but most perished as larvae (65%); only 23% emerged as viable adults. At this dose, no adult insects presented morphological deformities; only some interruptions of morphogenesis were observed in 38% of dead pupae. At dose of 15 μg/g, the mortality was 40%, and at 10 μg/g it was near the control. At this last dose, however, there were still occurrences of wing deformities or leg absence.

Assays with 4,5-epoxy-pentan-2-ol

In the experiments with doses of 0.45 and 0.23 mg of 4,5-epoxy-pentan-2-ol per gram of diet, all larvae died in less than or around 24 hours (Table 2.1). The dead larvae were either totally black or only from the head to the proleg area only; at the highest dose they were completely flaccid. At dose of 0.10 mg/g of epoxy alcohol, a mortality rate of 70% was observed, with 86% larvae dying within 48 hours; with 0.05 mg/g of the same product, the mortality rate was 40%, with 27% larvae dying within 48 hours. We also observed the blackening of dead larvae. In these last two doses, we also noted some deformities in adult butterflies like short wings, legs or antenna.

Assays with azadirachtin

Up to a dose of azadirachtin of 0.20 mg/g of diet, the mortality percentage was near the control (Table 2.1). However, 50 % of dead larvae showed characteristic blackening and one adult presented a shorter wing. With regard to these results, an experiment with azadirachtin by intrahemocoelic injection was performed. At a dose of 4 μg/g of larval body weight, we obtained a mortality rate of 100%. Nevertheless, the larvae made cocoons, but they died as larvae partly or totally black from 15 days to one month after the injection.

Assays with neem oil

All the arvae offered a diet containing 420 to 70 mg/g of Nature's neem oil were killed as larvae within one or two weeks without making a cocoon. Doses from 420 to 280 mg/g of this product, there was a repellent effect noted by the fact that larvae moved to the well-vented caps of the plastic cups. At doses of 28, 14 and 2.8 mg of neem oil per g of diet, the mortalities were respectively 60%, 60% and 30%. For all the doses tested, the larvae exhibited black spots (mainly at the lower doses) or were partially or totally black at the time of death. Proportionally to the lower doses (28 to 2.8 mg/g), emerging adults showed deformities as shorter or deformed wings (Figure 2.4 c) respectively at rates of 25, 12.5 and 8 % for 28, 14 and 2.8 mg/g.

Discussion

Our simpler epoxy-alcohols were more toxic for *G. mellonella* larvae than the commercial neem products and much more than the azadirachtin which killed the larvae only by injection. The epoxy-alcohols doses giving 100% mortalities were in the increasing order: glycidol, 4,5-epoxy-pentan-2-ol, and finally GDE (Figure 2.5). This was also confirmed by the estimated LD_{50} (lethal dose 50%) of each molecule (glycidol, 0.022 ppm; 4,5-epoxy-pentan-2-ol, 0.06 ppm; GDE, 0.14 ppm; Nature's neem oil™, 10.6 ppm). On the other hand, it was already noted that neither of the synthesized halves of the azadirachtin (decalin and hydroxyfuran acetal) has a growth regulatory activity and that they show only a modest antifeedant effect (Isman 1997). Our results confirm the importance of the epoxy-alcohol junction between the two parts of the molecule (Figure 2.2) for the biological activity.

We clearly observed a repellent effect with the neem product and GDE, as reported in other insects for azadirachtin (Gerard and Ruf 1995; Mulla and Su 1999). The same two components gave an antifeedant effect also reported for neem components (Blaney *et al.* 1990; Schmutterer 1990). As reported by Mordue *et al.* (1998), azadirachtin prevented feeding on Lepidoptera and caused a high mortality mainly due to starvation. In our study, the deaths associated with blackening were probably due to an insecticidal action by contact with the products. Black spots on Mexican bean beetle (*Epilachna varivestis*) treated with methanolic neem seed kernel extract were also reported by Schmutterer (1990). Azadirachtin caused also mortalities in insect as a result of its growth regulatory activity (Lowery and Isman 1994; Manal and Sehnal 2000; Mordue *et al.* 1998; Schmutterer 1990). The growth rate was notably reduced by glycidol in our results and metamorphosis was stopped with glycidol and GDE. There were deformities of the wings and legs with all our molecules, with neem commercial product and even with azadirachtin at a dose that did not kill the larvae. Wings malformations were reported by Schmutterer (1990); wing disc attacks (Schlüter 1987), as described by histological investigations, gave melanized degenerated cells resulting in the black spots (Schmutterer 1990).

We do not know if our molecules have exactly the same mode of action than the azadirachtin or the neem extract components. In the latter case, there is a complex interaction between insect and various components in the neem extract, possibly with synergism effects among them. Moreover, it is possible that some insects may degrade or detoxify the molecules which would allow their recovery, as it was reported for fungal toxins at sublethal doses applied by intrahaemocoelic injection (Jegorov *et al.* 1992). Also, some neem limonoids (nimbin and salannin) are unstable and can give photo-oxidation products as or more toxic than azadirachtin (Simmonds *et al.* 2004). Our molecules may undergo the same processes during the insect treatment. In this case, it will be useful to study their mode of action in a simpler system than the whole insect such as insect cell cultures with our molecules being labelled. The same kind of studies can be later conducted using various larval tissues to determine the targeted ones. Furthermore, one of our molecules, the glycidol, is classified as toxic for mammals by Aldrich Co. In the future, we should ramify the epoxy-alcohol fragment (Figure 2.2) to obtain a molecule as toxic for insect only and environmentally safe as azadirachtin but more stable.

Acknowledgements

We acknowledge the research support provided by the Fonds Québécois de la Recherche sur la Nature et les Technologies and the Foundation of the Université du Québec à Trois-Rivières (UQTR). We thank Mrs C. Descôteaux from UQTR for synthesis of the 4,5-epoxy-pentan-2-ol.

References

An M, Toochinda T, Bartlett PA, 2001. Five-membered ring analogues of shikimic acid. J. Org. Chem. 66, 1326-1333.

Aurrecoechea JM, Pérez E, Solay M, 2001. Synthesis of trisubstituted furans from epoxypropargyl esters by sequential SmI_2-promoted reduction-elimination and Pd(II)-catalyzed cycloisomerization. J. Org. Chem. 66, 564-569.

Blaney WM, Simmonds MSJ, Ley SV, Anderson JC, Toogood PL, 1990. Antifeedant effects of azadirachtin and structurally related compounds on lepidopterous larvae. Entomol. Exp. Appl. 55, 149-160.

Broughton HB, Ley SV, Slawin AMZ, Williams DJ, Morgan ED, 1986. X-ray crystallographic structure determination of detigloyldihydroazadirachtin and reassignment of the structure of the limonoid insect antifeedant azadirachtin. Journal of Chemical Society. 1, 46-47.

Bruce YA, Gounou S, Chabi-Olaye A, Smith H, Schulthess F, 2004. The effect of neem (*Azadirachta indica* A. Juss) oil on oviposition, development and reproductive potentials of *Sesamia calamitis* Hampson (Lepidoptera: Noctuidae) and *Eldana saccharina* Walker (Lepidoptera: Pyralidae). Agric. For. Entomol. 6, 223-232.

Butterworth JH, Morgan ED, 1968. Isolation of a substance that suppresses feeding in locusts. Journal of Chemical Society. Chemical Communications. 1, 23-24.

Butterworth JH, Morgan ED, 1971. Investigation of the locust feeding inhibition of the seeds of the neem tree, *Azadirachta indica*. J. Insect Physiol. 17, 969-977.

Finney DJ, 1971. Probit analysis. 3rd edition. Cambridge University Press, New York. 333 pages.

Garzon S, Strykowski H, Charpentier G, 1990. Implication of mitochondria in the replication of Nodamura virus in larvae of the Lepidoptera, *Galleria mellonella* (L.) and in suckling mice. Arch. Virol. 113, 165-176.

Gerard PJ, Ruf LD, 1995. Effect of a neem (*Azadirachta indica* A. Juss, Meliaceae) extract on survival and feeding of larvae of four keratinophagous insects. J. Stored Prod. Res. 31, 111-116.

Guan HP, Ksebati MB, Kern ER, Zemlicka J, 2000. Approaches to unsaturated analogues of nucleosides comprising four- and six-membered rings. J. Org. Chem. 65, 5177-5184.

Isman MB, 1997. Neem insecticides. Pesticide Outlook. 8, 32-38.

Jarvis AP, Johnson S, Morgan ED, 1998. Stability of the natural insecticide azadirachtin in aqueous and organic solvants. Pestic. Sci. 53, 217-222.

Jegorov A, Matha V, Hradec H, 1992. Detoxification of destruxins in *Galleria mellonella* L. larvae. Comp. Biochem. Physiol. 1, 227-229.

Koul O, 1999. Insect growth regulating and antifeedant effects of neem extracts and azadirachtin on two aphid species of ornamental plants. J. Biosciences. 1, 85-90.

Kraus W, Bokel M, Cramer R, Klenk A, Pöehnl H, 1985. Constituents of neem and related species. A revised structure of azadirachtin. Abstr. 3rd Int. Conf. Chem. Biotechnol. Biol. Act. Nat. Prod. 4, 446-450.

Ley SV, 1994. Synthesis and chemistry of the insect antifeedant azadirachtin. Pure Appl. Chem. 66, 2099-2102.

Ley SV, Anderson JC, Blaney WM, Morgan ED, Sheppard RN, Simmonds MSJ, Slawin AMZ, Smith SC, Williams DJ, Wood A, 1991. Chemistry of insect antifeedant from *Azadirachta indica* (Part 11): Characterization and structure activity relationships of some novel rearranged azadirachtins. Tetrahedron. 47, 9231-9246.

Ley SV, Denholm AA, Wood A, 1993a. The chemistry of azadirachtin. Nat. Prod. Rep. 109-157.

Ley SV, Lovell PJ, Slawin AMZ, Smith SC, Williams DJ, Wood A, 1993b. Chemistry of insect antifeedants from *Azadirachta indica* (Part 15): Degradation studies of azadirachtin leading to C8-C14 bond cleavage. Tetrahedron. 49, 1675-1700.

Lowery DT, Isman MB, 1994. Insect growth regulating effects of neem extract and azadirachtin on aphids. Entomol. Exp. Appl. 72, 77-84.

Manal MA, Sehnal F, 2000. Azadirachtin potentiates the action of ecdysteroid agonist RH-2485 in *Spodoptera littoralis*. J. Insect Physiol. 46, 267-274.

Medina P, Budia F, Del Estal P, Viñuela E, 2004. Influence of azadirachtin, a botanical insecticide, on *Chrysoperla carnea* (Stephens) reproduction: toxicity and ultrastructural approach. J. Econ. Entomol. 97, 43-50.

Mordue AJ, Simmonds MSJ, Ley SV, Blaney WM, Mordue W, Nasiruddin M, Nisbet AJ, 1998. Actions of azadirachtin, a plant allelochemical, against insects. Pestic. Sci. 54, 277-284.

Mulla MS, Su T, 1999. Activity and biological effects of neem products against arthropods of medical and veterinary importance. J. Am. Mosquito Contr. 15, 133-152.

Nishikimi Y, Iimori T, Sodeoka M, Shibasaki M, 1989. Synthetic studies of azadirachtin. Synthesis of the cyclic acetal intermediate in the naturally occurring form. J. Org. Chem. 54, 3354-3359.

Pflieger D, Muckensturm B, Robert PC, Simonis MT, Kienlen JC, 1987. Synthesis of the furo-pyran moiety of the azadirachtin. Tetrahedron Lett. 28, 1519-1522.

Schlüter U, 1987. Effects of azadirachtin on developing tissues of various insect larvae. In: Schumutterer H, Ascher KRS, Eds «Natural pesticides from the neem tree (*Azadirachta indica* A. Juss) and other tropical plants». Proceedings of the Third-International Neem Conference, Nairobi, Kenya, 10-15 july 1986, published by Deutsche Gesellschaft für Technische Zusammenarbeit (GTZ) GmbH, pp. 331-348.

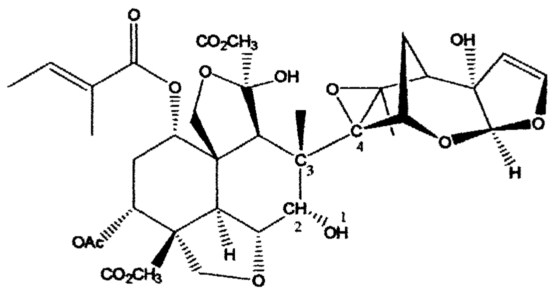
Schmutterer H, 1990. Properties and potential of natural pesticides from the neem tree, *Azadirachta indica*. Annu. Rev. Entomol. 35, 271-297.

Simmonds MSJ, Jarvis AP, Johnson S, Jones GR, Morgan ED, 2004. Comparison of anti-feedant and insecticidal activity of nimbin and salannin photo-oxidation products with neem (*Azadirachta indica*) limonoïds. Pest. Manag. Sci. 60, 459-464.

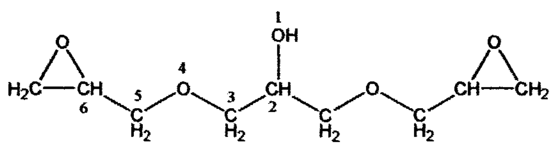
Su T, Mulla S, 1998. Ovicidal activity of neem products (azadirachtin) against *Culex tarsalis* and *Culex quinquefasciatus* (Diptera: Culicidae). J. Am. Mosquito Contr. 14, 204-209.

Su T, Mulla M, 1999. Oviposition bioassay responses of *Culex tarsalis* and *Culex quinquefasciatus* to neem products containing azadirachtin. Entomol. Exp. Appl. 91, 337-345.

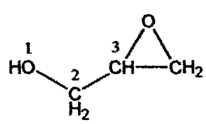
Watanabe H, Watanabe T, Mori K, 1996. Enantioselective synthesis of the tricyclic furan moiety of azadirachtin, a potent insect antifeedant. Tetrahedron. 52, 13939-13950.



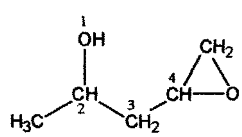
(I)



(II)



(III)



(IV)

Figure 2.1. The azadirachtin molecule (I) and the other molecules used in this study, glycerol diglycidyl ether (II), glycidol (III) and 4,5-epoxy-pentan-2-ol (IV).

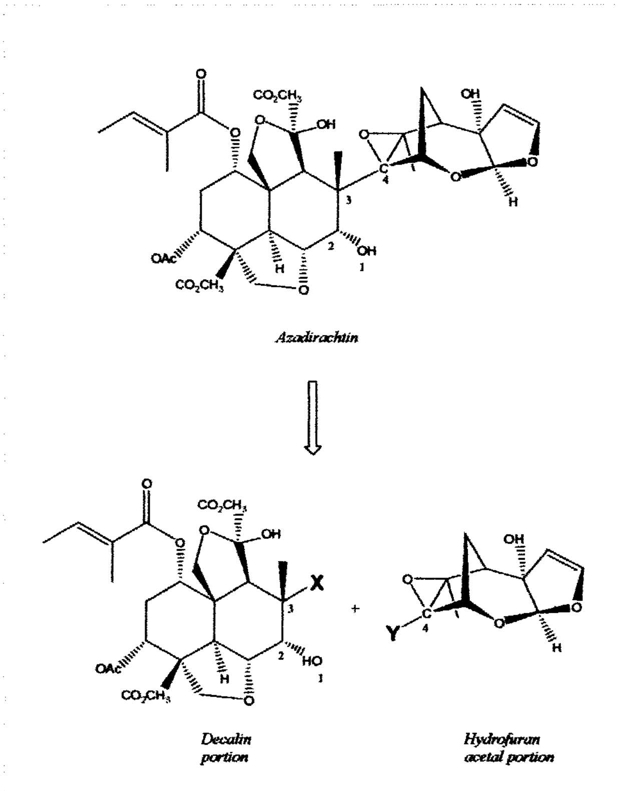


Figure 2.2. The decalin portion and the hydroxyfuran acetal moiety of the azadirachtin molecule.

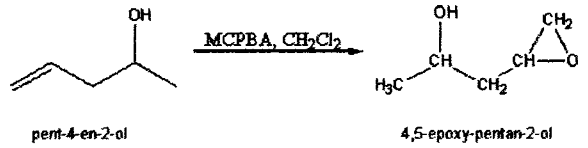


Figure 2.3. Reaction involved in the preparation of the 4,5-epoxy-pentan-2-ol.

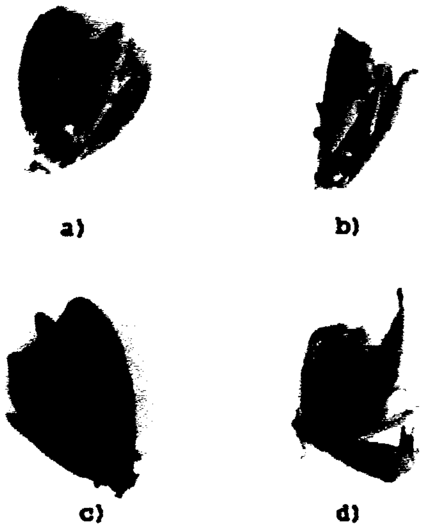


Figure 2.4. Morphological deformities of some specimens. Reference butterflies (a), specimen treated with GDE (b), specimen treated with the Nature's neem oil (c) specimen treated with glycidol. (d).

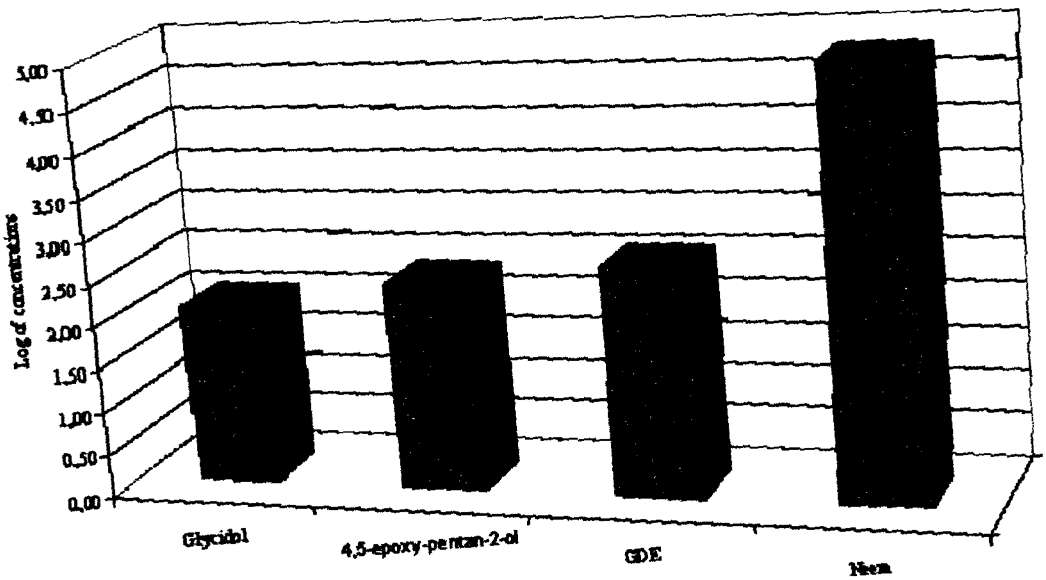


Figure 2.5. Minimum log concentrations of products giving 100% mortality.

Table 2.1. Effects of simpler epoxy-alcohols, azadirachtin and Nature's neem oil on larvae of *Galleria mellonella.*

Concentration (mg/g)	Dead larvae (%)	Days to cocooning	Days in cocoon	Days before Emergence
Control untreated[a]	21	3.7±2.9	15.0 ±3.3	19.0±4.4
Acetone control	20	1.3±0.8	14.0±4.3	15.6±3.1
Application of Glycerol diglycidyl ether				
0.40	100	ND	ND	ND
0.20	60	1.6±1.4	15.8±3.5	18.1±3.8
0.04	20	1.5±2.0	14.8±4.0	17.3±4.0
0.004	3	1.6±2.4	15.5±3.6	18.4±3.7
Application of Glycidol				
0.30	100	ND	ND	ND
0.15	100	ND	ND	ND
0.12	100	ND	ND	ND
0.06	80	0.8±0.3	11.8±1.2	12.6±0.9
0.03	77	1.1±0.4	12.2±4.1	13.4±1.8
0.015	40	4.1±1.7	15.3±4.4	20.4±4.2
0.010	20	3.2±0.6	13.4±3.7	17.6±3.4
Application of 4,5-epoxy-pentan-2-ol				
0.45	100	ND	ND	ND
0.23	100	ND	ND	ND
0.10	70	8.1±6.3	11.9±3.6	21.0±3.2
0.05	40	5.2±3.8	12.6±2.5	18.8±3.1
Azadirachtin				
0.20	17	4.0±2.0	17.7±3.9	21.7 ±4.1
0.004 injection	100	ND	ND	ND
Application of Nature's neem oil™				
70	100	ND	ND	ND
28	60	2.4±2.2	15.3±2.5	17.6±1.7
14	60	3.8±2.9	13.8±3.2	17.1±1.8
2.8	30	4.9±4.7	16.2±2.5	21.0±4.8

[a] :This control was done four times with 30 larvae per replicate.

CHAPITRE III

DEUXIÈME ARTICLE

Comparative effects of azadirachtin and simpler epoxy-alcohols on insect cell lines from Coleoptera, Diptera and Lepidoptera.

Carole Charbonneau,

Roland Côté

Guy Charpentier

Département de Chimie-Biologie, Université du Québec à Trois-Rivières, Trois-Rivières, Québec, Canada, G9A 5H7.

Short title: Effects of azadirachtin derivatives on insect cell lines.

Keywords: Glycerol diglycidyl ether, glycidol, 4,5-epoxy-pentan-2-ol, *Spodoptera frugiperda, Aedes triseriatus, Leptinotarsa decemlineata,* Cytotoxicity.

Abstract

In order to determine which molecular structure in azadirachtin is essential for insecticidal activity, we synthesized one epoxy-alcohol and tested it *in vivo*, together with two commercial similar molecules and azadirachtin. Following the results of these *in vivo* experiments showing a similar anti-insect activity of these molecules which contains these two functional groups (epoxy and alcohol) and azadirachtin, we tested them *in vitro* on three insect cell lines. The results presented in this paper allowed us to compare the *in vitro* effects of our molecules with those of azadirachtin described in the literature. First, we described the cytotoxic effects on three cell lines (Sf9, A.t. GRIP-1, and Ld-L1) of 1,3-(bis (2,3-epoxypropoxy)-propan-2-ol) (A), 2,3-epoxy-propan-1-ol (B), 4,5-epoxy-pentan-2-ol (C), azadirachtin and a neem formulation. We measured the cell growth rate and percentage of the mitotic figures with orcein staining. We observed, depending on the concentrations of the molecules, immediate and delayed cytotoxic effects as rounding and swelling of cells with vacuolization and in one case aggregation of the cells. The cytotoxicity of the molecules tested, in decreasing order of activity was azadirachtin followed by (A) then neem, (B) and (C). We also did a pretreatment of cells with juvenile hormone or insulin without any effect on cytotoxicity of our molecules. All molecules tested blocked cell proliferation to the same extent except for (C) on Sf9 cell line. An antimitotic effect was observed with azadirachtin and the three molecules tested on Ld-L1 cells. These last effects were observed by others authors with azadirachtin and related molecules on cell lines. The cytotoxicity of our molecules emphasizes the importance of the chemical structure between the two half moieties of azadirachtin for the biological activity. Further studies need to be done especially the

synthesis of ramified epoxy-alcohol molecules more complex and less toxic for nontarget organisms, but more stable than azadirachtin.

Introduction

Azadirachtin (I) (Figure 3.1), a plant-extracted molecule, is of great interest for pest control (Ley et al., 1993a). Azadirachtin was extracted from the neem tree *Azadirachta indica* (A. Juss), a plant from the Meliaceae family, by Butterworth and Morgan (1968, 1971) and identified as a tetranorterpenoid by Kraus et al. (1985) and Broughton et al. (1986). Unlike most chemical insecticides that are specific in their actions, azadirachtin produces various strong anti-insect effects (Schmutterer, 1990; Mordue et al., 1998; Su & Mulla, 1999) including repellent effects, feeding deterrence (antifeedancy) and growth regulation caused mainly by alterations of ecdysteroid and juvenile hormone concentrations. Some effects can also occur during the metamorphosis, such as impaired wing development (Schlüter, 1987). Negative effects on reproduction have also been reported, such as low oviposition rates (Medina et al., 2004; Mulla & Su, 1999), egg viability reduction (Bruce et al., 2004); more clearly, an ovicide effect (Su & Mulla, 1998) has been also observed. These pleotrophic effects imply complicated modes of action of the neem compounds.

The growth regulatory effects of these compounds (moulting delay or inhibition, deformities) are poorly known at tissues or cellular level (Nisbet et al., 2001). From experiments with the Mexican bean beetle (*Epilachna varivestis* Mulsant (Coleoptera: Coccinellidae: Epilachnini)), Schlüter (1987) concluded that mitosis in the L_4 wing disc epidermis is affected by azadirachtin. Furthermore, in cell culture experiments, it was demonstrated that azadirachtin binds to Sf9 nuclear fraction *in vitro* (Nisbet et al., 2001). This finding was further investigated on Sf9 cell culture by Salehzadeh et al. (2003)

showing an increment of mitotic figures with aberrations when treated with azadirachtin. These authors, assumed that azadirachtin acted on the mitosis by preventing the polymerisation of tubulin. Finally, Akudugu et al. (2001), with experiments about cytotoxicity of azadirachtin A in human glioblastoma cell lines, showed that azadirachtin can cause irreversible damage to cellular DNA directly or indirectly. These last authors pointed out the fact that there is a delay between the first damages to the target cells due to azadirachtin and the final effect that is cell death. During our experiments, the treated cells were observed for a week. On the contrary, Cohen et al. (1996) looking only for the early effects of limonoids on cells failed to observed the long term effects even with lower concentrations. They classified azadirachtin as a non-cytotoxic for Sf9 cells based on observations made only after 24 h of contact time.

Interestingly, some components of the neem extracts had biological activity on other types of cells like inhibitory effects on bacteria, action on malaria parasite, cytotoxicity effects against several human tumour cells and even a spermicidal effect (Cohen et al., 1996)

Attempts to synthesize the whole azadirachtin molecule have remained unsuccessful for over ten years (Ley, 1994; Ley et al., 1993b, 1991). While the two major portions of the molecule, the decalin portion and the hydroxyfuran acetal moiety, have been synthesized, neither has any insect growth regulatory (IGR) activity. We became interested in the link between those two portions since the study of simple molecules based on the epoxy-alcohol fragments of azadirachtin have not been reported yet. We tested three simpler epoxy-alcohol fragments of azadirachtin on *Galleria mellonella*

L.(Lepidoptera: Pyralidae: Galleriini) larvae and observed effects of these molecules similar to those of azadirachtin (Charbonneau et al., submitted to Journal of Applied Entomology). For a better understanding of the mode of action of our molecules (Glycerol diglycidyl ether, Glycidol and 4,5-epoxy-pentan-2-ol) (II, III, IV) (Figure 3.1), we studied their toxic effects on insect cell cultures from three order of insects (Coleoptera, Diptera and Lepidoptera).

Materials and Methods

Chemicals

Glycerol diglycidyl ether (GDE): 1,3-(bis (2,3-epoxypropoxy)-propan-2-ol) (II) (Figure 3.1), technical grade was purchased from Aldrich Co. (Oakville, Canada).

Glycidol: (2,3-epoxy-propan-1-ol) (III) (Figure 3.1), purity 96% was purchased from Aldrich Co. (Oakville, Canada).

The synthetic epoxy alcohol: 4,5-epoxy-pentan-2-ol (IV) (Figure 3.1), was prepared by the oxidation of 4-penten-2-ol with meta-chloroperbenzoic acid in dichloromethane as described by An et al. (2001), Aurrecoechea et al. (2001) and Guan et al. (2000). The synthetic product was purified by silica gel column chromatography and characterized using infrared and ^{13}C and ^{1}H nuclear magnetic resonance spectroscopy: The observed peak position, multiplicity and intensity are consistent with the calculated values for the proposed molecule.

The neem formulation was Nature's neem oil™ from Grotek Manufacturing Inc. (BC, Canada), and contained 2500 ppm of the active ingredient azadirachtin.

The 95% pure azadirachtin (I) (Figure 3.1) was purchased from Sigma-Aldrich Canada Ltd (Oakville, Canada).

Cell Culture

Sf9 cells, derived from *Spodoptera frugiperda* J. E. Smith (Lepidoptera: Noctuidae: Amphipyrini) pupal ovarian tissue (Vaughn et al., 1977), were obtained from Dr. Belloncik (INRS-Institut Armand-Frappier, Qc, Canada) and were cultured in Mitsuhashi-Maramorosch culture medium (M.M.) (Gibco™, N-Y, USA).

Aedes triseriatus Say (Diptera: Culicidae: Culicini) cells A.t. GRIP-1 (Charpentier et al., 1995) derived from the mosquito species collected in the Trois-Rivières area (Qc, Canada) were started from neonate larval tissues. Cells were grown in M.M. culture medium.

Ld-L1 cells, initiated from the Colorado potato beetle *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae: Doryphorini) larval haemocytes (Charpentier et al., 2002), were grown in Ex-cell 400 culture medium (JRH Biosciences, KS, USA).

Each cell line was cultured in 50 mL tissue culture flasks (Corning, N-Y, USA) containing the culture medium plus 10% foetal calf serum (Gibco™, N-Y, USA) and 1% penicillin/streptomycin at 10 000 µg/ml (Gibco BRL, N-Y, USA). Cultures were maintained at 3×10^5 to 1×10^6 cells/ml at 28 °C and cells were subcultured every two weeks.

Furthermore, Sf9, A.t. GRIP-1, Ld-L1 subcultures were treated with juvenile hormone-III (purity 75%, Sigma-Aldrich, Oakville, Canada) and insulin (Sigma-Aldrich, Oakville, Canada) because of a putative reversion by hormones of the effects of azadirachtin simpler moieties as described for azadirachtin effects on some insects

(Schlüter, 1987). Each hormone was diluted in the appropriate culture medium (M.M. or Ex-cell 400) and 250 μL of a solution of 40 ppm were added to the culture flask containing 5 ml of culture cell at $3x10^5$ to $1x10^6$ cells/ml. Every 2 weeks for 6 to 8 weeks before the assays, the subcultures of these cell lines were retreated as above.

Toxicity assays

Cytotoxicity tests: Cells from tissue culture flasks of the different cell lines (Sf9, A.t. GRIP-1, Ld-L1) and the three subcultures of the cell lines treated with the juvenile hormones or insulin were detached from the plastic by gentle pipetting, and 150 μl of a cell suspension of 50 000 to 60 000 cells/ml were inoculated into each well of a 96-well tissue culture microplate (Corning, N-Y, USA). The microplates were incubated 24 h at 28 °C before the addition of 50 μl of the different compounds at various concentrations. The GDE only was first dissolved in 200 μl of acetone, diluted in culture medium and filtered-sterilized using Millipore 0,45μm type HA (Millipore Ltd. Canada). The mock-treated cells received either an equivalent amount of the appropriate culture medium, or for the assays with GDE, a 10% acetone solution diluted in the culture medium. The cell cultures were observed under microscope 24 h and seven days after treatments. Experiments were done in triplicate.

Cell counts: Culture flasks of the three cell lines (Sf9, A.t. GRIP-1, Ld-L1) were incubated 24 h at 28 °C before the addition of the different compounds at various concentrations. The cells were exposed for seven days, and were counted using a haemocytometer (American Optical Company, N-Y, USA) after 48 h and seven days post-treatments. Three experiments were performed for each cell line and the results were expressed as the ratio of cell count at time zero to the cell count at each time after treatment.

Chromosome staining: The cell pellet obtained from centrifugation (10 min at low speed in IEC Clinical Centrifuge) of 5 ml of treated or mock culture flasks after seven days of treatment was washed with distilled water and then allowed to swell for 10 min. After another centrifugation, the cells were fixed with glacial acetic acid and methanol (1:3) for 10 min. After the last centrifugation, they were stained with orcein (2 % in a 30% acetic acid solution in distilled water) for 24 h. A drop of stained cell suspension was put on a microscope slide and covered with a lamella, which was pressed in order to spread the chromosomes. The percentage of cells showing metaphase was observed for 100 cells (Charpentier et al., 2002).

Results

Cytotoxicity tests. We first determined the limit concentrations giving an observable effect on the three cell cultures (Sf9, A.t. GRIP-1, Ld-L1) with each compound (Table 3.1). The limit dilution was better seen for some cell cultures and compounds seven days after the treatment. The juvenile hormones or insulin treatments did not affected the effects of the compounds tested (Table 3.1). The sensitivity of the three cell lines seemed to be the same for each individual compound. The cell lines were most modified by azadirachtin (0.7 to 7.2 ppm) followed by GDE (307 to 410 ppm) and neem (418 to 558 ppm) and in decreasing order by glycidol (1393 to 2230 ppm) and 4,5–epoxy-pentan-2-ol (1700 to 2126 ppm).

Under the microscope, Ld-L1 cells treated with different compounds and acetone showed a rounding of the cells and the cytoplasm appeared to be granulated (Figure 3.2). The control cells were of two types, round type and spindle type, with a clear cytoplasm; the extracellular background of the culture did not contain remains of cells (Figure 3.2 A). This last observation was also made for acetone control. In comparison with these control cell cultures and other treated cell cultures, azadirachtin-treated cell culture showed an important background of cell remains (Figure 3.2 C). In some cases, there were vacuoles in the cell cytoplasm as for cells treated with acetone control (Figure 3.2 B) and, with 4,5-epoxy-pentan-2-ol. In these cases, we noted an aggregation of cells as with azadirachtin (Figure 3.2 C) and GDE. In the case of glycidol, some cells were very deformed (Figure 3.2 D).

The mosquito cells (A.t. GRIP-1) normally rounded in culture showed a granulated cytoplasm only for treated cells. Generally, except with azadirachtin, the treated cells became smaller. With azadirachtin, the mosquito cells became swollen and 6 to 10 times larger than the normal cell (Figure 3.3); furthermore, there were no remains in the extracellular background contrary to Ld-L1 cells treated with azadirachtin. For glycidol, we observed as in the case with Ld-L1 cells, that there was nearly no intact cell.

Sf9 cells, normally rounded in shape, showed with the treatments a granulated cytoplasm, cell remains in the background and vacuoles. These effects were more obvious with the glycidol treatment of these cells.

Cell counts. All cell lines growth rate at seven days post-treatment were drastically diminished for the four compounds tested and for the acetone control (Figure 3.4). The azadirachtin showed a marked effect on cell growth rate for the three cell lines (Figure 3.5) with Sf9, being the most sensitive. On the opposite, the 4,5-epoxy-pentan-2-ol gave for Sf9 cells an increase of the growth rate (1.60 times) but a decrease for the two others cell lines. The glycidol also gave a decrease of cell growth but more important on Ld-L1 than on A.t. GRIP-1 and Sf9. Because of the effect of acetone (to dissolve GDE) alone on cell growth rate, the results for GDE were difficult to interpret for Ld-L1 and Sf9 cell lines. With GDE, there was more lysis of the cells than with acetone only. For A.t. GRIP-1 cell line, however, there was an important decrease in the growth rate compared to the control with acetone on the same cells.

Number of mitotic cells. For Ld-L1 cell line, the percentages of mitotic cells were significantly greater with all compound treatments than those of the two controls

(Figures 3.6 and 3.7). These percentages were in the same range of magnitude. The Sf9 cell line presented this phenomenon of mitotic arrest only with the azadirachtin treatment. However, the A.t. GRIP-1 cell line did not show any effect of mitotic arrest.

Discussion

Results show that depending of the concentrations of the molecules, we observed an immediate and delayed cytotoxic effects. This pointed out the fact that azadirachtin or related molecules could damage the DNA of the cells and that cell death takes at least 40 h to be observed (Akudugu et al., 2001). The immediate cytotoxic effects were the rounding and swelling of cells was also described by Reed & Majumdar (1998) for the effects of azadirachtin on Sf9 cells. Vacuoles were seen in Ld-L1 cells and Sf9 treated with 4,5-epoxy-pentan-2-ol and glycidol, and this phenomenon can be compared to the extensive nimbolide-related vacuolization described by Cohen et al. (1996) on N1E-115 neuroblastoma (mouse) cell line. With azadirachtin and GDE on Ld-L1 cells, there was an aggregation of cells never described by other authors. As in the studies of Salehzadeh et al. (2002), the *Spodoptera frugiperda* cell line (Sf9) was the most sensitive to azadirachtin-related molecules among the insect cell lines we tested. The cytotoxicity of the tested compounds were in the decreasing order, azadirachtin followed by GDE and neem, and glycidol and 4,5-epoxy-pentan-2-ol. The most complex molecules were the most toxic, contrary to what we obtained when they were tested *in vivo* on *Galleria mellonella* larvae (Charbonneau et al., submitted to Journal of Applied Entomology).

The azadirachtin-related molecules blocked cell proliferation to the same extent except for 4,5-epoxy-pentan-2-ol on Sf9 cell line. This effect on cell growth was also described by several authors with azadirachtin or related molecules (Akudugu et al., 1998; Cohen et al., 1996; Reed & Majumdar, 1998). An antimitotic effect was clearly visible with azadirachtin and the three molecules tested on Ld-L1 cells. Salehzadeh et al. (2003) also observed the same antimitotic effect of azadirachtin on Sf9 cell line.

In order to thwart the effects of azadirachtin or related molecules on cells, we submitted cell lines to a pretreatment with juvenile hormone or insulin. These pretreatments failed to produce any difference on the treatment effects or on the concentrations producing these effects. This is not surprising since the three insect cell lines originated from holometabolus insects for which the *in vivo* treatment with insect hormones did not prevent injected azadirachtin from impairing complete molt (Schlüter, 1987). Even though we knew this fact before; we did these experiments because the *in vivo* or *in vitro* effects of azadirachtin could have been quite different.

The cytotoxicity of our molecules demonstrates their biological activities similar to those of azadirachtin *in vitro*, and all these molecules contain the two functional groups epoxy and alcohol found in azadirachtin between the two half moieties. While the two major portions of the molecule, the decalin portion and the hydroxyfuran acetal moiety, have been synthesized, neither had any insect growth regulatory activity and only modest levels of antifeedant action (Isman, 1997). Furthermore, these two half moieties of azadirachtin were tested *in vitro* on Sf9 and were less effective for reducing growth than the whole molecule (Salehzadeh et al., 2002). There was no synergistic effect on cells treated with a mixture of the two moieties (Salehzadeh et al., 2002).

We will have to determine separately the effect on cells of each chemical functional group in our molecules; epoxy and alcohol, in order to specify the contribution of each functionality to the biological activities. Further studies need to be done, especially the synthesis of ramified epoxy-alcohol molecules, more complex and less toxic for nontarget organisms but more stable than azadirachtin. Also, experiments should be performed on competition for the binding site of azadirachtin as it was done by Nisbet et al. (2001) for azadirachtin analogues. Studies to determine if there is a putative binding or target sites and their possible localizations at cellular level deserve more investigations. Our new molecules may have other interesting properties allowing medical applications: like neem components, they can possibly display antibacterial, antitumor, spermicidal and antimalarial activities (Cohen et al., 1996).

Acknowledgements; We acknowledge the research support provided by the Fonds Québécois de la Recherche sur la Nature et les Technologies and the Foundation of the Université du Québec à Trois-Rivières (UQTR). We thank Mrs C. Descôteaux from UQTR for synthesis of the 4,5-epoxy-pentan-2-ol.

References

Akudugu J, Gäde G & Böhm L (2001) Cytotoxicity of azadirachtin A in human glioblastoma cell lines. Lifes Sciences 68: 1153-1160.

An M, Toochinda T & Bartlett PA (2001) Five-membered ring analogues of shikimic acid. Journal of Organic Chemistry 66: 1326-1333.

Aurrecoechea JM, Pérez E & Solay M (2001) Synthesis of trisubstituted furans from epoxypropargyl esters by sequential SmI_2-promoted reduction-elimination and Pd(II)-catalyzed cycloisomerization. Journal of Organic Chemistry 66: 564-569.

Broughton HB, Ley SV, Slawin AMZ, Williams DJ & Morgan ED (1986) X-ray crystallographic structure determination of detigloyldihydroazadirachtin and reassignment of the structure of the limonoid insect antifeedant azadirachtin. Journal of Chemical Society 1: 46-47.

Bruce YA, Gounou S, Chabi-Olaye A, Smith H & Schulthess F (2004) The effect of neem (*Azadirachta indica* A. Juss) oil on oviposition, development and reproductive potentials of *Sesamia calamitis* Hampson (Lepidoptera: Noctuidae) and *Eldana saccharina* Walker (Lepidoptera: Pyralidae). Agricultural and Forest Entomology 6: 223-232.

Butterworth JH & Morgan ED (1968) Isolation of a substance that suppresses feeding in locusts. Journal of Chemical Society. Chemical Communications 1: 23-24.

Butterworth JH & Morgan ED (1971) Investigation of the locust feeding inhibition of the seeds of the neem tree, *Azadirachta indica*. Journal of Insect Physiology 17: 969-977.

Charbonneau C, Côté R & Charpentier G (2006) Effects of azadirachtin and simpler epoxy-alcohols on survival and behaviour of *Galleria mellonella* (Lepidoptora) larvae. Journal of Applied Entomology. Accepted manuscript JEN-05-2006-0168.

Charpentier G, Belloncik S, Ducros G, Fontenille D, Tian L & Quiot JM (1995) Establishment and characterization of three cell lines from *Aedes triseriatus* (Diptera : culicidae). Journal of Medical Entomology 32: 793-800.

Charpentier G, Tian L, Cossette J, Léry X & Belloncik S (2002) Characterization of cell lines developed from the Colorado potato beetle, *Leptinotarsa Decemlineata Say* (Coleoptera: Chrysomelidae) *In vitro* Cellular & Developmental Biology-Animal 38: 73-78.

Cohen E, Quistab GB & Casida JE (1996) Cytotoxicity of nimbolide, epoxyazadiracdione and other limonoids from neem insecticide. Life Sciences 58: 1075-1081.

Guan HP, Ksebati MB, Kern ER & Zemlicka J (2000) Approaches to unsaturated analogues of nucleosides comprising four- and six-membered rings. Journal of Organic Chemistry 65: 5177-5184.

Isman MB (1997) Neem insecticides. Pesticide Outlook. 8: 32-38.

Kraus W, Bokel M, Cramer R, Klenk A & Pöehnl H (1985) Constituents of neem and related species. A revised structure of azadirachtin. Abstract. 3rd Int. Conf. Chem. Biotechnol. Biol. Act. Nat. Prod. 4, 446-450.

Ley SV (1994) Synthesis and chemistry of the insect antifeedant azadirachtin. Pure and Applied Chemistry. 66: 2099-2102.

Ley SV, Anderson JC, Blaney WM, Morgan ED, Sheppard RN, Simmonds MSJ, Slawin AMZ, Smith SC, Williams DJ & Wood A (1991) Chemistry of insect antifeedant from *Azadirachta indica* (Part 11): Characterization and structure activity relationships of some novel rearranged azadirachtins. Tetrahedron. 47: 9231-9246.

Ley SV, Denholm AA & Wood A (1993a) The chemistry of azadirachtin. Natural Product Reports. 109-157.

Ley SV, Lovell PJ, Slawin AMZ, Smith SC, Williams DJ & Wood A (1993b) Chemistry of insect antifeedants from *Azadirachta indica* (Part 15): Degradation studies of azadirachtin leading to C8-C14 bond cleavage. Tetrahedron 49: 1675-1700.

Medina P, Budia F, Del Estal P & Viñuela E (2004) Influence of azadirachtin, a botanical insecticide, on *Chrysoperla carnea* (Stephens) reproduction: toxicity and ultrastructural approach. Journal of Economic Entomology 97: 43-50.

Mordue AJ, Simmonds MSJ, Ley SV, Blaney WM, Mordue W, Nasiruddin M & Nisbet AJ (1998) Actions of azadirachtin, a plant allelochemical, against insects. Pesticide Science 54: 277-284.

Mulla MS & Su T (1999) Activity and biological effects of neem products against arthropods of medical and veterinary importance. Journal of the American Mosquito Control Association 15: 133-152.

Nisbet AJ, Mordue AJ, Grossman RB, Jennens L, Ley SV & Mordue W (2001) Characterization of azadirachtin binding to Sf9 nuclei *in vitro*. Archives of Insect Biochemistry and Physiology 46: 78-86.

Reed E &Majumdar SK (1998) Differential cytotoxic effects of azadirachtin on *Spodoptera frugiperda* and mouse cultured cells. Entomologia Experimentalis et Applicata 89: 215-221.

Salehzadeh A, Akhkha A, Cushley W, Adams RLP, Kusel JR & Strang RHC (2003) The antimitotic effect of the neem terpenoid azadirachtin on cultured insect cells. Insect Biochemistry and Molecular Biology 33: 681-689.

Salehzadeh A, Jabbar A, Jennens L, Ley SV, Annadurai RS, Adams, R & Strang RHC (2002) The effects of phytochemical pesticides on the growth of cultured invertebrate and vertebrate cells. Pest Management Science 58: 268-276.

Schlüter U (1987) Effects of azadirachtin on developing tissues of various insect larvae. In: Natural pesticides from the neem tree (*Azadirachta indica* A. Juss) and other tropical plants. Proceedings of the Third-International Neem Conference, Nairobi, Kenya, 10-15 july 1986 (ed. by Schumutterer H & Ascher KRS). Deutsche Gesellschaft für Technische Zusammenarbeit (GTZ) GmbH. 331-348.

Schmutterer H (1990) Properties and potential of natural pesticides from the neem tree, *Azadirachta indica*. Annual Review of Entomology 35: 271-297.

Su T & Mulla S (1998) Ovicidal activity of neem products (azadirachtin) against *Culex tarsalis* and *Culex quinquefasciatus* (Diptera: Culicidae). Journal of the American Mosquito Control Association 14: 204-209.

Su T & Mulla M (1999) Oviposition bioassay responses of *Culex tarsalis* and *Culex quinquefasciatus* to neem products containing azadirachtin. Entomologia Experimentalis et Applicata 91: 337-345.

Vaughn JL, Goodwin RH, Tompkins GJ & Mc Cowley P (1977) The establishment of two insect cell lines from the insect *Spodoptera frugiperda* (Lepidoptera : Noctuidae). *In vitro* Cellular & Developmental Biology 13: 223-217.

Figure legends

Figure 3.1 The azadirachtin molecule (I) and the other molecules used in this study, glycerol diglycidyl ether (II), glycidol (III) and 4,5-epoxy-pentan-2-ol (IV).

Figure 3.2 Microscopical observations at seven days of *Leptinotarsa decemlineata* cell line (Ld-L1) treated with azadirachtin and simpler epoxy-alcohols.

A) Ld-L1 control cells were round or spindle-shaped and presented a clear cytoplasm and extracellular background. B) Acetone control showed a rounding of cells and vacuoles (black arrow). C) Cells treated with 7.2 ppm of azadirachtin exhibited aggregations of cells in clumps (thick white arrows). D) 2230 ppm glycidol-treated cells. E) Glycerol diglycidyl ether treatment (410 ppm). F) Cell treated with 1913 ppm of 4,5-epoxy-pentan-2-ol.

Figure 3.3 Microscopical observations of A.t. GRIP-1 *Aedes triseriatus* cell line with azadirachtin seven days post-treatment.

A) A.t. GRIP-1 control cells and B) cells treated with 7.2 ppm of azadirachtin showing a swelling of cells 6 to 10 times the size of control cells.

Figure 3.4 Growth rate measured at seven days of Ld-L1, Sf9 and A.t. GRIP-1 cell lines treated with azadirachtin and simpler epoxy-alcohols.

The various concentrations of the different compounds used in this experiment were determined by the limit concentrations giving an effect in the cytotoxicity assays.

Figure 3.5 Growth rate at seven days post-treatment of Ld-L1, Sf9 and A.t. GRIP-1 cell lines treated with azadirachtin and simpler epoxy-alcohols without the mock-treated cells.

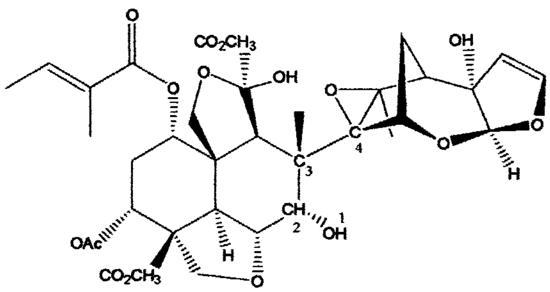
Figure 3.6 Mitotic cell percentages at seven days, for Ld-L1, Sf9 and A.t. GRIP-1 cell lines treated with azadirachtin and simpler epoxy-alcohols.

The various concentrations of the different compounds were the same as in growth rate assays cell count assays.

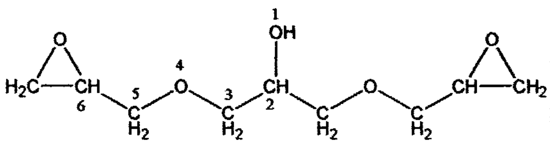
Figure 3.7 Microscopical observations of mitotic cells of *Leptinotarsa decemlineata* cell line (Ld-L1) treated with the 4,5-epoxy-pentan-2-ol.

A) Ld-L1 control cells. B) cells treated with 1913 ppm of 4,5-epoxy-pentan-2-ol: in both figures, cell with mitotic arrest are indicated with black arrows.

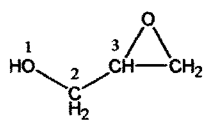
Figure 3.1



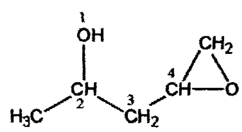
(I)



(II)



(III)



(IV)

Figure 3.2

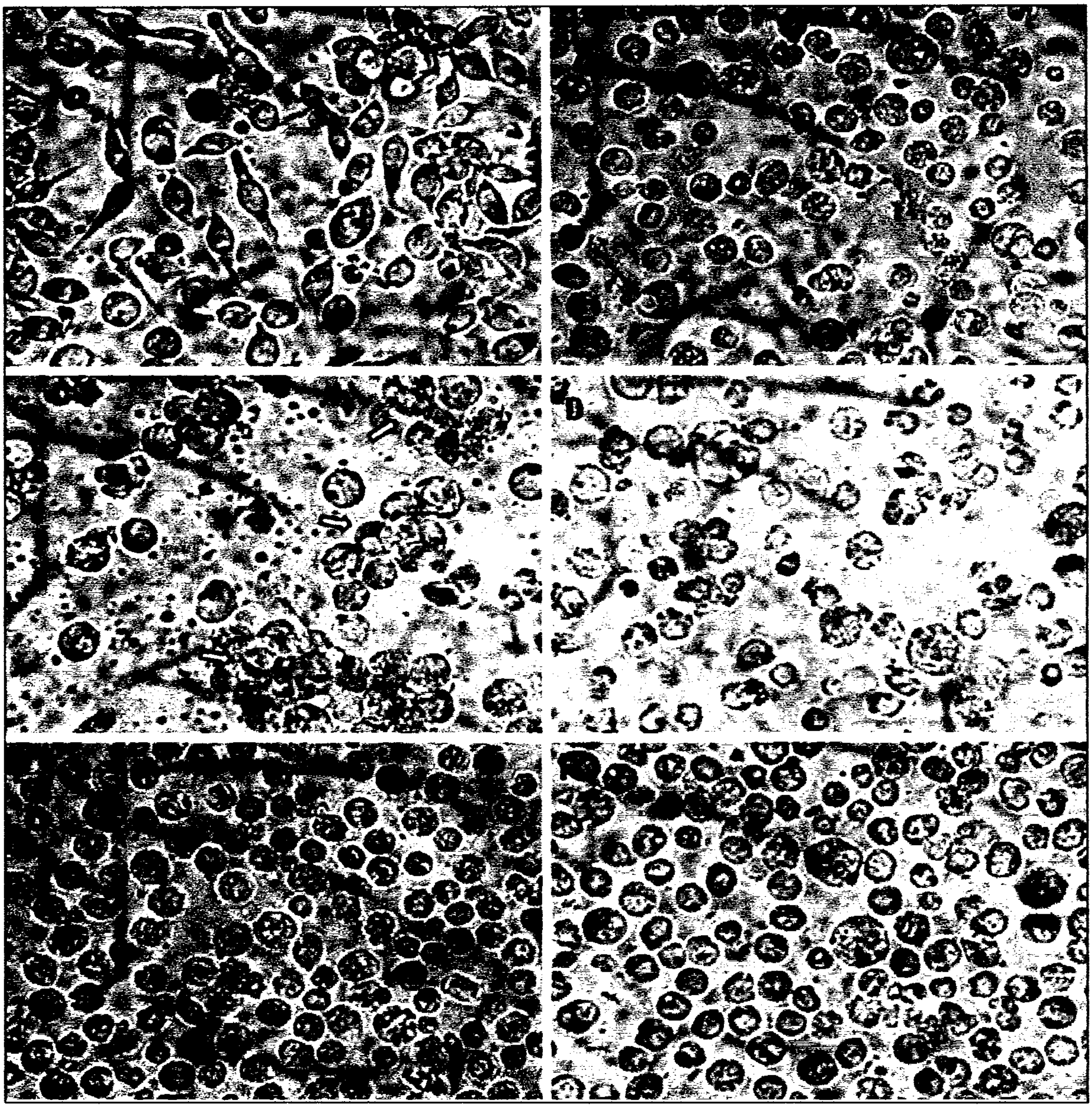


Figure 3.3

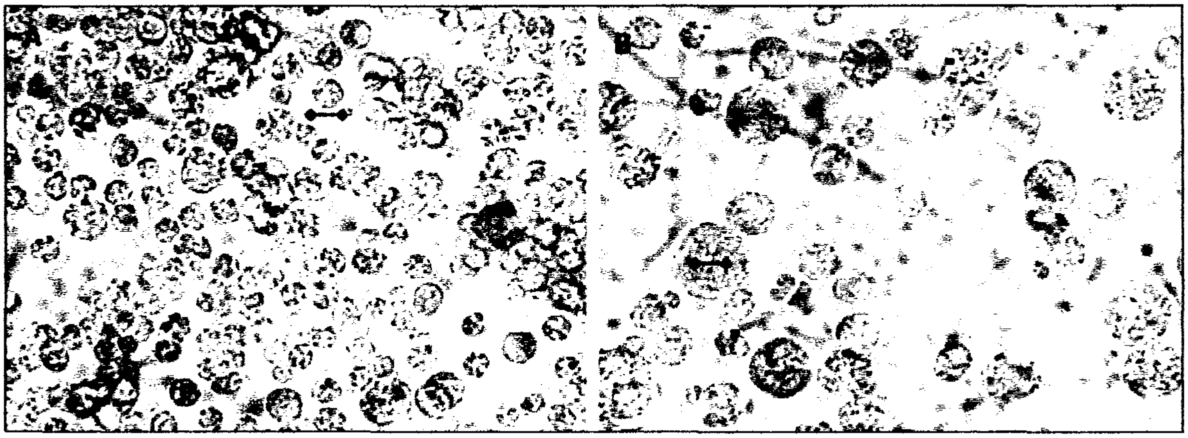


Figure 3.4

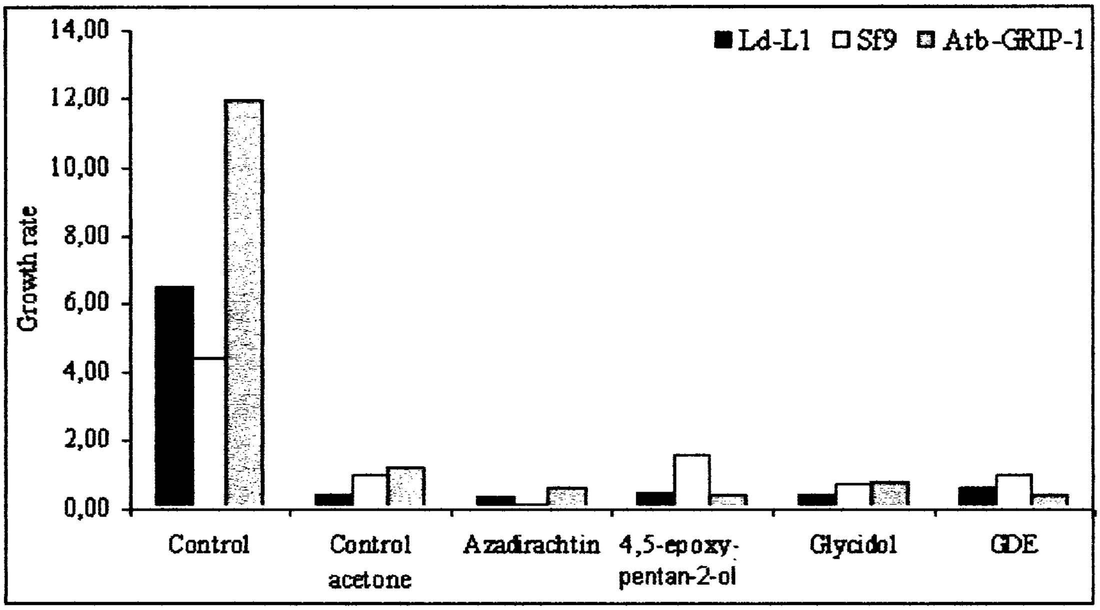

■ Ld-L1 □ Sf9 ▒ Atb-GRIP-1
Growth rate
14,00
12,00
10,00
8,00
6,00
4,00
2,00
0,00
Control
Control acetone
Azadirachtin
4,5-epoxy-pentan-2-ol
Glycidol
GDE

Figure 3.5

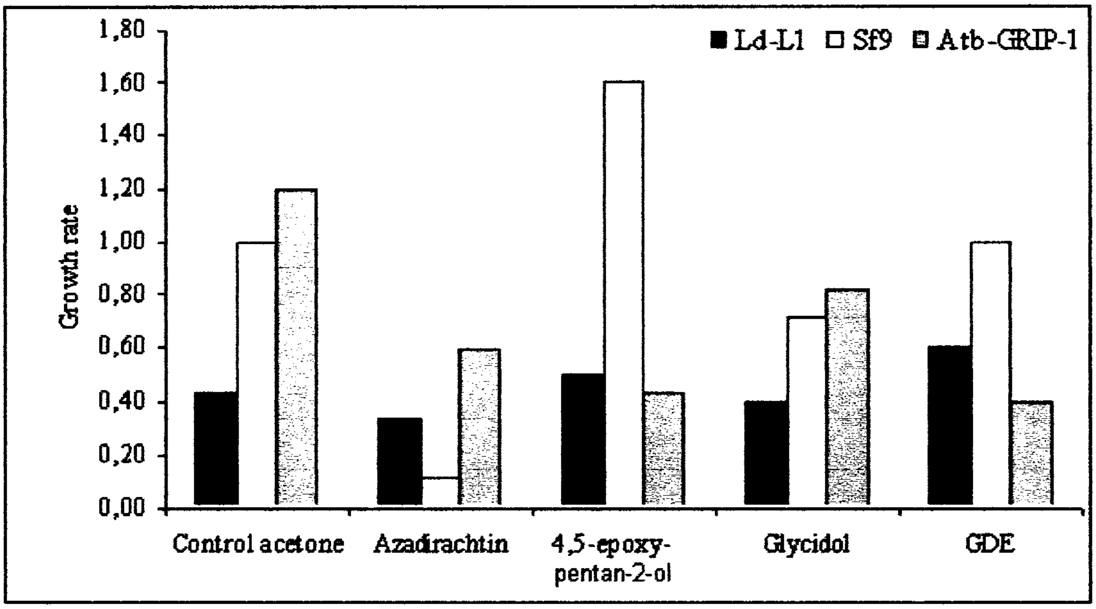
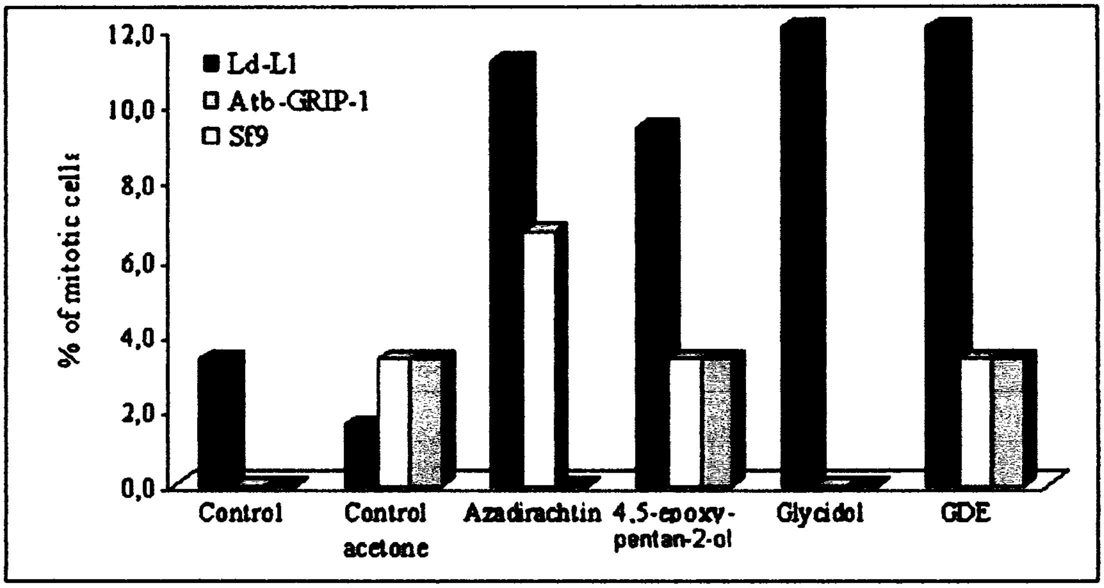

■ Ld-L1 □ Sf9 ▤ Atb-GRIP-1
Growth rate
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Control acetone
Azadirachtin
4,5-epoxy-pentan-2-ol
Glycidol
GDE

Figure 3.6



12,0
10,0
8,0
6,0
4,0
2,0
0,0
% of mitotic cells
Ld-L1
Atb-GRIP-1
Sf9
Control
Control acetone
Azadirachtin
4,5-epoxy-pentan-2-ol
Glycidol
GDE

Figure 3.7

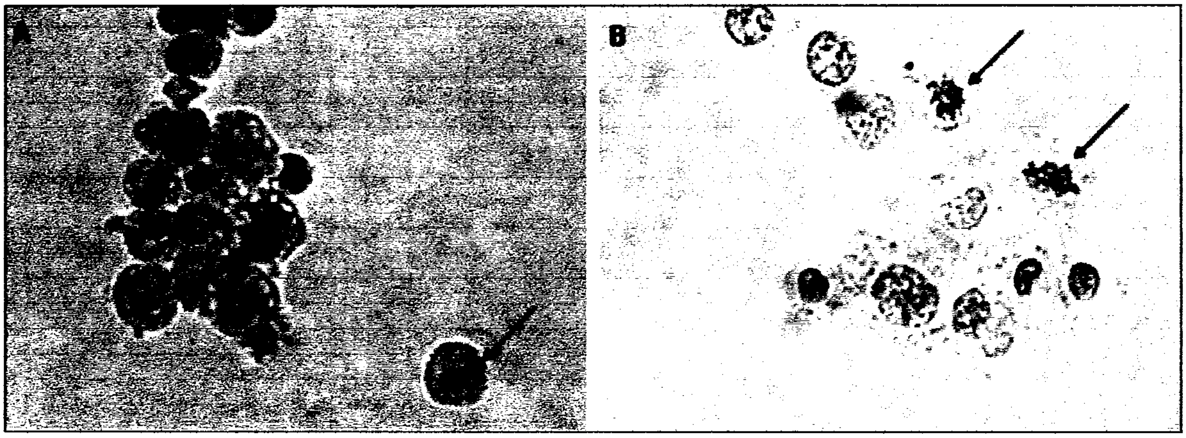


Table 3.1 Limit concentrations of azadirachtin and simpler epoxy-alcohols (ppm) giving an effect on Sf9, A.t. GRIP-1 and Ld-L1 cell cultures after 24 h and seven days.

	Untreated cells			Cells treated with insulin			Cells treated with juvenile hormone		
24 h	Sf9	A.t. GRIP-1	Ld-L1	Sf9	A.t. GRIP-1	Ld-L1	Sf9	A.t. GRIP-1	Ld-L1
Azadirachtin	7.2	7.2	7.2	ND	ND	ND	ND	ND	ND
Nature's neem oil (ppm azadirachtin))	558	465	418	558	558	489	558	558	558
Glycerol diglycidyl ether	366	523	492	430	819	351	615	615	351
Glycidol	1858	2230	2322	1393	2322	2523	2322	2322	2322
4,5-epoxy-pentan-2-ol	2126	> 2126	1913	1913	> 2126	1700	2126	> 2126	1700
7 days	Sf9	A.t. GRIP-1	Ld-L1	Sf9	A.t. GRIP-1	Ld-L1	Sf9	A.t. GRIP-1	Ld-L1
Azadirachtin	0.70	7.2	7.2	ND	ND	ND	ND	ND	ND
Nature's neem oil (ppm azadirachtin))	558	418	418	558	418	418	349	418	489
Glycerol diglycidyl ether	307	400	410	369	615	351	307	460	351
Glycidol	1393	2230	2230	697	> 2126	1488	929	> 2126	1488
4,5-epoxy-pentan-2-ol	1700	2126	1913	1913	2126	1700	1913	2126	1700

>: At this maximum concentration tested no effect was observed.

ND: Not Done.

ANNEXES

Manuscripts submitted to ***Entomologia Experimentalis et Applicata***, or any part of the work described therein, must be original and must not be simultaneously under consideration for publication elsewhere. Work previously published in abstract form should be referred to in the introduction. The Editors cannot accept responsibility for damage to or loss of papers submitted to them.

Online

Entomologia Experimentalis et Applicata strongly encourages online submission of manuscripts at http://mc.manuscriptcentral.com/eea. Submission online enables the quickest possible review and allows online manuscript tracking. Manuscript submission online can be in Word document (.doc), Rich Text Format (.rtf), or PostScript (.ps). Each of these file formats will be automatically converted to PDF for reviewing. Figures can be embedded in the native word processor file or may be uploaded separately in one of the following formats: GIF (.gif), JPEG (.jpg), TIFF (.tif), EPS (.eps). Figures uploaded separately in these file formats will be automatically converted to small jpegs for reviewing. Other file formats may be uploaded, but these will not be converted to an easily opened format and Editors and Referees will require the necessary software to view these files.

Full upload instructions and support are available online from the submission site via the Get Help Now button. You can also email for online submission support. When prompted online, please submit your covering letter or comments to the Editor-in-Chief including one or two sentences describing the contents of the paper and its relevance to the focus of the journal. Please do not duplicate your submission by submitting online and by post.

Please note that following acceptance of your manuscript for publication in *Entomologia Experimentalis et Applicata*, you will be required to supply your manuscript and figures on disk (for disk instructions see Disk Submission below).

Author material archive policy

Please note that unless specifically requested, **Blackwell Publishing will dispose of all hardcopy or electronic material submitted two months after publication**. If you require the return of any material submitted, please inform the editorial office or production editor as soon as possible if you have not yet done so.

Presentation of manuscripts

Manuscripts, including the references, should be double-spaced, line numbered, and written in grammatically correct English. The title page should state the title of the paper, the names and addresses of the authors, a short title for running headlines, the full address of the corresponding author and up to 12 key words, which should not duplicate words in the title. An Abstract of not more than 300 words should include the reason why the work was carried out, the key techniques, the results and the major conclusions. The abstract should contain the full scientific name, followed by that of the family and tribe of the subject insect(s), along with the full name(s) of food plants, chemicals, etc. The Latin names of insects as well as plants should be followed by the name of the describer. Original articles should include, in the following order, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Figure legends (starting on a separate sheet), Tables. The text should preferably be in MS Word (PC or Mac format) and the illustrations must be submitted according to the Blackwell Publishing digital illustration standards (see below). The Editors reserve the right to adjust style to certain standards of uniformity.

The SI system of units should be used for all scientific and laboratory data; temperature should be expressed in degrees Centigrade. A table of commonly used SI units and their symbols is available to view by clicking the following link: http://www.blackwellpublishing.com/pdf/eea_ifa.doc

Technical Notes

A short manuscript (up to four pages in print) that describes a new technique or rearing method, or substantial improvements of existing methods, or that presents data on the bio-activity of a novel substance may be submitted as a Technical Note. These short manuscripts should not have an abstract, and can be organized either along the lines of a regular manuscript or without subdivisions. Authors may also consider combining the results and discussion sections. Technical Notes should not present incomplete or preliminary data sets.

References

References in the text. References should be in the Harvard style, i.e. citations appear in the form (Smith, 1990; Smith & Jones, 1991, Smith et al., 1992), (Smith et al., 1990) or if only the year is given in brackets, there should be no comma after the author name, e.g., The results of recent work by Smith et al. (1992).... References to papers having three or more authors should give the name of the first author only, followed by et al.

Reference list. References should be listed alphabetically at the end of the article and should conform to the formats of the following examples. Journal titles should be given in full.

Journal article:

Dicke M & van Loon JJA (2000) Multitrophic effects of herbivore-induced plant volatiles in an evolutionary context. Entomologia Experimentalis et Applicata 97: 237-249.

Book:

Gullan PJ & Cranston PS (1999) The Insects. An outline of entomology. 2nd edn. Blackwell Science, Oxford, UK.

Book chapter:

Shapiro M (1986) In vivo production of baculoviruses. The Biology of Baculoviruses, Vol. 2: Practical Application for Insect Control (ed. by RR Grandos & BA Federici) CRC Press, FL, USA, pp. 31-62.

Where a DOI is included in a reference, it should be cited as follows:

Mazmanian SK, Ton-That H & Schneewind O (2001) Sortase-catalysed anchoring of surface proteins to the cell wall of *Staphylococcus aureus*. Molecular Microbiology 40: 1049-1057. doi:10.1046/j.1365-2958.2001.02411.x

or alternatively if it has not yet been allocated to an issue:

Mazmanian SK, Ton-That H & Schneewind O (2001) Sortase-catalysed anchoring of surface proteins to the cell wall of *Staphylococcus aureus*. Molecular Microbiology. doi:10.1046/j.1365-2958.2001.02411.x

References should be checked carefully to make sure that all references given in the text (and no others) appear in the list of references and that the spelling of authors names and the dates are correct.

Illustrations

Illustrations must be in a finished form ready for reproduction. All lettering in the figures, where possible, should be in '**Arial**' font. Subdivided figures should be lettered A, B, etc. in the top left hand corner. The maximum figure size is 175 x 224 mm. Magnifications should be indicated by scale bars. Labelling should be clear and sized appropriately for the intended final size that the figure is intended to appear in the journal. Figures should be referred to in the text as (Figure 10). The authors name, the figure number and orientation should be shown on the back of each illustration. Authors may be asked to reduce the number of figures if some are considered to be inessential.

Do not send original copies of drawings with the submitted manuscript. The Editor will request the original figures after the manuscript has been accepted for publication.

Electronic artwork. We would like to receive your artwork in electronic form. Save line art (vector graphics), uncompressed if possible, in Encapsulated PostScript (.eps) format and bitmap files (halftones or photographic images) in Tagged Image Format Files (.tif).

Detailed information on the submission of electronic artwork can be found at http://www.blackwellpublishing.com/bauthor/journal.asp

Exclusive Licence Form

Authors will be required to sign an Exclusive Licence Form (ELF) for all papers accepted for publication. Signature of the ELF is a condition of publication and papers will not be passed to the publisher for production unless a signed form has been received. Please note that signature of the Exclusive Licence Form does not affect ownership of copyright in the material. (Government employees need to complete the Author Warranty sections, although copyright in such cases does not need to be assigned). After submission authors will retain the right to publish their paper in various medium/circumstances (please see the form for further details). To assist authors an appropriate form will be supplied by the editorial office. Alternatively, authors may like to download a copy of the form.

OnlineOpen

OnlineOpen is a pay-to-publish service from Blackwell that offers authors whose papers are accepted for publication the opportunity to pay up-front for their manuscript to become open access (i.e. free for all to view and download) via the Blackwell Synergy website. Each OnlineOpen article will be subject to a one-off fee of £1250 (equivalent to $2500) to be met by or on behalf of the Author in advance of publication. Upon online publication, the article (both full-text and PDF versions) will be available to all for viewing and download free of charge. The print version of the article will also be branded as OnlineOpen and will draw attention to the fact that the paper can be downloaded for free via the Blackwell Synergy service.

Any authors wishing to send their paper OnlineOpen will be required to complete the combined payment and copyright licence form available from our website at: http://www.blackwellpublishing.com/pdf/EEA_OOF.pdf

(Please note this form is for use with OnlineOpen material ONLY.)

Once complete this form should be sent to the Production Editor along with the rest of the manuscript materials at the time of acceptance or as soon as possible after that (preferably within 24 hours to avoid any delays in processing). Do not inform the Editorial Office that you intend to publish your paper OnlineOpen before acceptance.

The copyright statement for OnlineOpen authors will read:

© [date] The Author(s)

Journal compilation © [date] The Netherlands Entomological Society /Blackwell Publishing Ltd

Colour Illustrations

It is the policy of Entomologia Experimentalis et Applicata for authors to pay the full cost for the reproduction of their colour artwork, with the exception of colour figures in Mini Review articles.

Therefore, please note that if there is colour artwork in your manuscript when it is accepted for publication, Blackwell Publishing require you to complete and return a colour work agreement form before your paper can be published. This form can be downloaded as a PDF* from the internet. If you are unable to download the form, please contact the Production Editor at the address below and they will be able to email or FAX a form to you.

Once completed, please return the form to the Production Editor at the address below:

Production Editor
Entomologia Experimentalis et Applicata
Blackwell Publishing Ltd
101 George Street
Edinburgh
Scotland
EH2 3ES
Fax:+44 (0) 131 226 3803
Email:eea@oxon.blackwellpublishing.com

Any article received by Blackwell Publishing with colour work will not be published until the form has been returned.

* To read PDF files, you must have Acrobat Reader installed on your computer. If you do not have this program, this is available as a free download from the following web address:

http://www.adobe.com/products/acrobat/readstep2.html

Disk Submission

When supplying your manuscript on disk, please follow these instructions and return the disk and hard copy of your manuscript to the Editorial Office.

- Final version of the hard copy and the floppy disk must be the same. It is essential that the disk contains any last-minute changes.
- File name(s) should be given with the disk, with separate file names for: text, references, tables, figure captions.
- If possible, files should be supplied in both word processor and ASCII formats.
- Be consistent. Use the same presentation for all headings, etc. that are to appear the same in the finished printing.
- Tables may not be set from disk, and it is essential that an adequate hard copy is supplied.
- Advise us about any matter that you think could cause a problem.
- Do not use the carriage return (enter) at the end of lines within a paragraph.
- Disks will not be returned to authors.

Proofs

The corresponding author will receive an email alert containing a link to a web site. A working e-mail address must therefore be provided for the corresponding author. The proof can be downloaded as a PDF (portable document format) file from this site. Acrobat Reader will be required in order to read this file. This software can be downloaded (free of charge) from the following web site:

http://www.adobe.com/products/acrobat/readstep2.html.

This will enable the file to be opened, read on screen and printed out in order for any corrections to be added. Further instructions will be sent with the proof. Hard copy proofs will be posted if no e-mail address is available. Excessive changes made by the author in the proofs, excluding typesetting errors, will be charged separately.

Offprints

A PDF offprint of the online published article will be provided free of charge to the corresponding author, and may be distributed subject to the Publisher's terms and conditions. Paper offprints of the printed published article may be purchased if ordered via the method stipulated on the instructions that will accompany the proofs. Printed offprints are posted to the correspondence address given for the paper unless a different address is specified when ordered. Note that it is not uncommon for printed offprints to take up to eight weeks to arrive after publication of the journal. For order enquiries please email: offprint@cosprinters.com

NEW: Online production tracking is now available for your article through Blackwell's Author Services.

Author Services enables authors to track their article - once it has been accepted - through the production process to publication online and in print. Authors can check the status of their articles online and choose to receive automated e-mails at key stages of production. The author will receive an e-mail with a unique link that enables them to register and have their article automatically added to the system. Please ensure that a complete e-mail address is provided when submitting the manuscript. Visit www.blackwellpublishing.com/bauthor for more details on online production tracking and for a wealth of resources including FAQs and tips on article preparation, submission and more.

List of abbreviations

ANCOVA	analysis of covariance (not small caps)
ANOVA	analysis of variance (not small caps)
°C	Celsius/centigrade
ca. (roman)	circa/approx.
c.p.m.	counts per minute
centrifugal force: use ***g*** rather than r.p.m.	
cv	cultivar
day	not abbreviated
d.f.	degrees of freedom
Department	(*not* Dept)
Dr	doctor (*not* dr)
e.g.,	always followed by a comma
et al.	(roman)
Experiment	(*not* Expt)
F value	not italic
g	centrifugal force; Use instead of r.p.m.
g	gram
h	hour
i.e.,	always followed by a comma
in vitro	(roman)
in vivo	(roman)
l	litre
L16:D8	photoperiod indication
	(not 16L:8D)
Mu symbol	roman
min	minute
n	number, not in italics
The Netherlands	
ns	non-significant
P value	Always roman (not italic)
r.h.	relative humidity
r.p.m.	revolutions per minute - do not use! Supply value in ***g*** force
room temperature	(do not abbreviate to RT)
s	second

SD	standard error
SEM	standard error of the mean
U-test	U not italic
UK	(*not* Scotland, England, Wales, Northern Ireland, nor GB)
USA	
vs.	versus
vice versa	(roman, no hyphen)
vol/vol	volume by volume
week	not abbreviated
year	not abbreviated

The ***Journal of Applied Entomology*** publishes original articles on current research in applied entomology, including mites and spiders in terrestrial ecosystems. Research on stored product pests will be also accepted. The language of publication is English. Authors whose native language is not English, are requested to consult a native English speaker with their paper. All manuscripts are evaluated in a peer-review process. Authors are welcome to recommend potential reviewers for their papers.

1. Manuscript submission

Please submit your manuscript online at **http://mc.manuscriptcentral.com/jappent** Manuscripts that do not strictly adhere to the instructions given in the appropriate boxes will not be accepted for further processing.

Manuscripts are submitted to *Journal of Applied Entomology* online, i.e. electronically, from the corresponding author's JEN Manuscript Central account. You will need your files in an electronic format, an Internet connection, and a user ID and password for the site. To begin a new submission go to **http://mc.manuscriptcentral.com/jappent** and log in or create an account to get your user ID and password. Full instructions are provided on the site. If you encounter technical problems when submitting your manuscript, please contact Journal of Applied Entomology Manuscript Central Support e-mail:

Support@ScholarOne.com
phone: (434) 817-2040 ext.167 USA

2. Types of manuscripts

Original contributions
Short communications

Advances in methodology
Mini reviews
Perspectives
Book reviews

3. Manuscript requirements

External form

Original articles should not exceed 6,000 words, and short communications should not exceed 1,000 words. Scientific names of genus, species, and subspecies should be formatted in italics. The full name of species and subspecies must be used when first mentioned in a paper, and its abbreviation used thereafter.

Any abbreviations that are not listed must be explained in the text. The text body should be properly structured according to the requirements of the category (original articles: abstract, keywords, short title, introduction, material and methods, results, discussion, acknowledgements, references, figures and tables).

Manuscript title

Please limit the title to 16 words.

Manuscript short title

Please provide a short title, a maximum of 50 characters in length.

Abstract

The abstract should be easy to understand and not require reference to the body of the paper. Only very important results should be presented numerically in the abstract; it must not contain any abbreviations or statistical details. The abstract should not exceed 300 words.

Keywords

Provide a maximum of seven key words to the abstract for both original contributions and review articles. There should be no overlapping with the title.

Short title

Provide a short title of maximum 8 words.

Units of measurement, abbreviations, and nomenclature

All measurement specifications must follow the SI system. Other abbreviations should only be employed if they are recognised internationally. All biological and chemical terms used should follow current international nomenclature. Substances or reagents obtained commercially must include the name and address of the manufacturer or supplier when first mentioned in the text. Concentrations of solutions may be given as molar or normal concentrations. All other concentrations are expressed as percentages.

References

Each reference should conform to the examples given below, with author names separated only by a comma as shown. Full stops should not be used in the forename intitials. The name of a journal should be given in the abbreviated form, strictly following the codes given in

http://library.caltech.edu/reference/abbreviations/

The references should be listed in alphabetical order according to the surname of the first author and should give the full title of the paper. Anonymous contributions should be cited at the beginning of the bibliography.

a. From journals:

Kocsis L, Grannett J, Walker MJ, 2002. Performance of Hungarian phylloxera strains on *Vitis riparia* rootstocks. J. Appl. Entomol. 126, 567-571.

b. From books or other non-serial publications:

Chapman RF, 1998. The insects - Structure and function. 4th edition. Cambridge University Press, Cambridge.

c. From reference book contributions:

Zlotkin E, 2001. Insecticides affecting voltage-gated ion channels. In: Biochemical site of insecticide action and resistance. Ed. by Ishaaya I, Springer, Berlin, Heidelberg, 43-76.

d. Unpublished work should only be cited when it has been accepted for publication, and then together with the name of the journal in which it has been accepted.

For example:

Kocsis L, Grannett J, Walker MJ, 2002. Performance of Hungarian phylloxera strains on *Vitis riparia* rootstocks. J. Appl. Entomol. (in press).

e. online citation:

Beckleheimer J, 1994. How do you cite URLs in a bibliography? [WWW document]. URL http://www.nrlssc.navy.mil/meta/bibliography.html.

In the running text, citations should be made as in the following examples: Beroks and Bode (1999), or (Richter 1991, 1993; Starck and Schneider 2001). Up to two authors, give the names; for more than two authors, give the name of the first author followed by "*et al.*"

Figures and Tables

Figures and Tables should be uploaded separately. Please number figures and tables consecutively in the order in which they occur in the text. All figures must be provided in reproducible form. If figures or tables from other publications are used, the source must be given. Please mark the position of tables/figures in the text.

Tables should be created using the table function in MS Word or the tabulator in other programmes.

Line drawings should only be contour drawings without halftones (photo, shades of grey). Please do not use patterns; rough hatching is possible. Graphs with an x and y axis should not be enclosed in frames.

Photographs or prints
Authors have to meet the full cost of colour reproduction. For specific instructions read the "*Colour Work Agreement Form*" during the submission

4. Copyright Assignment

Authors are no longer required to assign copyright in their paper. Instead authors are required to assign the exclusive licence to publish their paper to Blackwell Verlag and the *Journal of Applied Entomology*. Assignment of the exclusive licence is a condition of publication and papers will not be passed to the publisher for production unless licence has been assigned. (Papers subject to government or Crown copyright are exempt from this requirement). Please download the Exclusive Licence Form here.

5. Proof correction and offprints

The corresponding author will receive proofs via e-mail as an Acrobat PDF file which should be reviewed and returned without delay using the resubmission instructions at

http://mc.manuscriptcentral.com/jappent

The corresponding author will receive a pdf file as an offprint. Additional offprints can be ordered on the form attached to the proofs. Offprints ordered at a later date will be subject to an extra charge.

6. Book reviews

At the end of each issue, book reviews are usually published in the fields of ecology and entomology. At the beginning of each book review, precise bibliographic details must be given in the following form: Family name, followed by the Christian name initials of the author of the book; the book's title; which edition (if not the first); place of publication; name of publisher; year of publication; number of pages, figures and tables; ISBN; binding and retail price.

The editor and publisher endeavour to publish all accepted articles for the journal as timely as possible. They request all authors to carefully observe the recommendations laid down in the 'Author Guidelines', so that publication of the Journal may be carried out with the highest possible efficiency, and to everyone's satisfaction.

The editor and publisher endeavour to publish all accepted articles for the journal as timely as possible. They request all authors to carefully observe the recommendations laid down in the 'Author Guidelines', so that publication of the Journal may be carried out with the highest possible efficiency, and to everyone's satisfaction.